In vitro REGENERATION OF *Dioscorea alata* WITH ANTHOCYANIN AND ANTIMICROBIAL ACTIVITY

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FACULTY OF SCIENCE
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In vitro REGENERATION OF *Dioscorea alata* WITH ANTHOCYANIN AND ANTIMICROBIAL ACTIVITY

ABSTRACT

An efficient protocol was developed for rapid propagation and regeneration of the *Dioscorea alata* L. locally known as 'ubi badak' because of their rhinoceros-shaped tubers. In the present study, direct and indirect regeneration were induced to produce plantlets of *Dioscorea alata* L. For direct regeneration explants (leave, stem and node) were cultured on MS media supplemented with various combinations and concentrations of BAP and NAA to induce microshoots and roots formation. The optimum medium for microshoots formation was MS medium supplemented with 1.0 mg/l BAP and 0.5 mg/l NAA with the number of microshoot is 23.07±0.44 from node explant. While for root formation, MS medium supplemented with 2.0 mg/l NAA is the optimum medium with mean number of roots formation 17.40±0.58. For indirect regeneration, microtubers formation, callus induction, somatic embryogenesis and synthetic seeds production were studied. MS medium supplemented with 1.0 mg/l NAA and1.0 mg/l BAP, is the best medium for microtubers formation with 15.57±0.18 mean number of microtubers. MS medium supplemented with 1.0 mg/l BAP and mg/l 1.0 NAA was the best medium for microtubers germination with percentage of germination 76.67±0.08 %. Callus induction was obtained on MS media supplemented with various concentration of 2,4-D, NAA, TDZ, BAP, myo-inositol and activated charcoal. Node explant cultured in MS medium supplemented with 2.0 mg/l 2,4-D and 1.0 mg/l BAP added with 0.5 mg/l myo-inositol was the best condition for optimum callus production. Callus were then identified whether it is embryogenic or non embryogenic using double staining method. Embryogenic callus was stained in red and non embryogenic callus was stained in blue. Embryogenic callus was then subculture onto solid and liquid somatic embryos induction medium. MS medium supplemented with 2.5 mg/l 2,4-D combine with 1.0 mg/l BAP is the best

medium for somatic embryos formation from 0.5 cm of embryogenic callus. Synthetic seeds were created by encapsulated propagules (microshoots, node and stem) with 16 different solution of sodium alginate and Calcium chloride dehydrate. In this study, propagules were successfully encapsulated in 2.0 to 5.0 % sodium alginate solution and harden in 25.0 to 100.0 mM Calcium chloride dehydrate solution. The beads varied morphologically with respect to texture, shape and transparency with different combinations of sodium alginate solution and calcium chloride. 3% sodium alginate harden in 100 µM Calcium chloride dehydrate was found to be the best encapsulation solution. Complete plantlets obtained from direct and indirect regeneration were then transferred to greenhouse. Plantlets response positively when acclimatized in garden soil (combination of black soil and red soil at ratio 2 to 1) with 93.33±0.09 % of survival rate. Callus exposed to different photoperiod produced different amount of anthocyanin. The total anthocyanin content was calculated using pH differential method. The highest (295.21 ± 0.20 mg cya-3-glu / 100 g FW) anthocyanin content was obtained from callus exposed to 16 hours light and 8 hours dark. Antimicrobial activity studies reveal that this plant response positively against bacteria (*Staphylococcus aureus*, *Staphylococcus epidermis*, *Escherichia coli* and *Salmonella sp.*) and fungi (*Penicillium sp.*, and *Mucor sp*.) negative effect against *Aspergilus niger*and *Fusarium sp.*

Keywords: Regeneration, microtubers, synthetic seed, antimicrobial.

REGENERASI *in vitro* DALAM *Dioscorea alata* DENGAN AKTIVITI ANTHOCYANIN DAN ANTIMIKROBIAL

ABSTRAK

Satu protokol yang efisien telah dihasilkan untuk regenerasi dan propagasi pesat pokok *Dioscorea alata* L. atau dikenali sebagai 'ubi badak' kerana ubinya berbentuk seperti badak. Dalam kajian ini regenerasi secara terus dan tidak telah dijalankan untuk memperolehi anak pokok *Dioscorea alata* L. Untuk regenerasi secara terus, eksplan (daun, batang, dan nod) dikultur dalam media MS yang ditambah dengan pelbagai kombinasi dan kepekatan hormon BAP dan NAA untuk menggalakkan pembentukan pucuk dan akar. Media yang optimum untuk pembentukan pucuk adalah media MS yang ditambah 1.0 mg/l BAP dan 0.5 mg/l NAA dengan pembentukan pucuk sebanyak 23.07±0.44 daripada explan node. Manakala untuk pebentukan akar, media MS ditambah 2.0 mg/l NAA adalah optimum dengan pembentukan akar sebanyak 17.40±0.58. Untuk regenerasi secara tidak terus, pembentukan microtuber, induksi kalus, penghasilan Embriogenesis somatic dan penghasilan biji benih tiruan diperhatikan. Media MS yang ditambah dengan1.0 mg/l NAA dan1.0 mg/l BAP, adalah media terbaik untuk pembentukan microtuber dengan purata bilangan microtuber 15.57±0.18. Media MS yang dibekalkan dengan 1.0 mg/l BAP dan mg/l 1.0 NAA adalah media terbaik untuk percambahan microtuber dengan peratusan percambahan 67±0.08. Induksi kalus diperolehi dalam media MS yang ditambah dengan pelbagai kepekatan hormone 2,4-D, NAA, TDZ, BAP, myo-inositol dan activated charcoal. Explan nod yang dikultur dalam media MS yang dibekalkan dengan 2.0 mg/l 2,4-D dan 1.0 mg/l BAP ditambah 0.5 mg/l myo-inositol adalah kondisi yang optimum untuk pembentukan kalus. Kalus kemudiannya dikenalpasti sama ada embriogenik atau pun tidak menggunakan teknik pewarnaan berganda. Kalus embriogenik akan diwarnakan dengan warna merah dan

kalus bukan embriogenik akan diwarnakan dengan warna biru. Selepas itu, kalus embriogenik di subkultur ke atas media penggalak pembentukan embrio somatik pepejal dan cecair. Media MS yang dibekalkan dengan 2.5 mg/l 2,4-D dan 1.0 mg/l BAP adalah media terbaik untuk pembentukan embrio somatic daripada , 0.5 cm kalus embryogenic. Biji benih tiruan dicipta dengan menkpsulkan propagul (pucuk micro, batang dan node) dengan 16 solusi berbeza sodium alginate dan Calcium chloride dehydrate. Dalam kajian ini, propagul berjaya dikapsulkan dengan 2.0 ke 5.0 % sodium alginate dan dikeraskan dalam 25.0 hingga 100.0 mM Calcium chloride dehydrate. Biji yang dihasilkan mempunyai morfologi berbeza dari segi tektur, bentuk dan ketulasan dengan kombinasi berbeza sodium alginate dan calcium chloride. 3% sodium alginate yang dipejalkan dalam 100 μM Calcium chloride dehydrate merupakan media penkapsulan terbaik. Plantlet yang diperolehi semasa regenerasi secara terus dan tidak, kemudian dipindahkan ke rumah hijau. Plantlet memberi respon positif bila di aklimitasi pada tanah kebun (gabungan tanah hitam dan merah dengan nisbah 2 kepada 1) dengan 93.33±0.09 % kadar keterushidupan. Kalus yang didedahkan kepada photoperiod berbeza menghasilkan kandungan anthocyanin yang berbeza. Jumlah kandungan anthocyanin dikira menggunakan kaedah perbezaan pH. Kandungan anthocyanin yang paling tinggi (295.21 ± 0.20 mg cya-3-glu / 100 g FW) diperolehi daripada kalus yang didedahkan kepada 16 jam cahaya dan 8 jam gelap. Kajian aktiviti antimikrobial menunjukkan, pokok ini memberi tindak balas positif kepada bakteria, (*Staphylococcus aureus*, *Staphylococcus epidermis*, *Escherichia coli* dan *Salmonella sp.*) dan fungi (*Penicillium sp.* dan *Mucor sp*.) dan tindak balas negative kepada *Aspergilus niger* dan *Fusarium sp.*

Kata kunci: Regenerasi, mikrotuber, biji benih tiruan, antimicrobial.

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BAP	Benzylaminopurine
2, 4-D	2, 4-Dichlorophenoxyacetic acid
HCL	Hidrochloric acid
Kinetin	6-Furfurylaminopurine
kPa	Kilo Pasca
mg/l	Milligram per Liter
min	Minute
MS	Murashige and Skoog
NAA	Naphthalene acetic acid
NaOH	Sodium Hydroxide
TDZ	Thiadiazuron
v/v	Volume per volume
w/v	Weight per volume
mg/l	Miligram per litre
ANOVA	Analysis of variance
LSD	Least significant differences
DMRT	Duncan Multiple Range Test
SE	Standard error

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Introduction

Dioscorea alata L. or known as "purple yam" is a yam species. It is a vigorously twining herbaceous vine with massive underground tubers. Aerial tubers formed in leaf axils, elongated up to 10 cm x 3 cm in size. The tubers are rough with bumpy surfaces. This plant is cultivated throughout the subtropical and tropical regions of the world for their edible tubers which constitute a staple food for many people in these geographical regions. Originated from the Asian countries, *Dioscorea alata* has been acknowledged to human since prehistoric times. *Dioscorea alata* has many different names include water yam, greater yam and winged yam. In Malaysia it is known as ubi badak. In traditional medicine, *Dioscorea alata* has been used as a treatment for fever, laxative and vermifuge, tumors, inflamed hemorrhoids, leprosy and gonorrhea. In modern medicine, the tubers are reported to have anticlastogenic effect (Wang et al., 2011), antihypertensive (Liu et al, 2009), bone protective (Chen et al., 2008) and immunostimulatory effect (Shang et al., 2007). *Dioscorea alata* is also valued for its starch content and used in a variety of desserts, as well as a flavor for ice cream, milk, Swiss rolls, tarts, cookies, cakes, and other pastries. *Dioscorea alata* is sometimes grown in gardens for its ornamental value.

Tubers of *Dioscorea alata* have deep purple colour verified to be anthocyanin pigment and well known for their antioxidant activity. For the past decade, anthocyanins was recognize as an alternatives for synthetic dyes and food colourant because of their attractive colours. Colour plays a significant role in our satisfaction of food stuffs, it is appreciated both for its aesthetic role and as a basis for the assessment of quality. Colour also gives viewable signs to enhance flavor identification and taste limits, influencing food preference, food acceptability and food choice. With expanding public worry about

the safety of synthetic colourant, natural pigment extracts are accepting more prominent noticeable quality. Anthocyanins are natural, nontoxic pigments in charge of the blue, red and purple colours of fruits, vegetables, flowers and other plant tissues. Existing evidence demostrate that the anthocyanin are non-harmful and non-mutagenic, yet have positive therapeutic properties (Saija, 1994), antioxidant and antimicrobial properties.

Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species (ROS) such as singlet oxygen, superoxide, peroxyl radicals, and peroxy nitrite which result in oxidative stress. It is gradually being understood that many of the modern human diseases started because of the oxidative stress. This can lead to oxidative damage of protein and nucleic acid, cellular damage and disease progression. Deficiency of antioxidant to reduce the surplus of reactive free radicals can cause different diseases like cancer, inflammatory disorder, neurodegenerative and etc. Hence, recent studies are centred on plant having high free radical quenching properties as a substitute to dietary intake of synthetic antioxidant which could cause genotoxicity and carcinogenicity at high concentration (Labo et al., 2010). Recently, natural antioxidant and natural food colourant from plant pigments in the form of carotenoids and flavonoids (anthocyanins) are high in demand due to their potential in wellbeing advancement and disease prevention and their improve safety and consumer acceptability.

Consumption of synthetic antioxidants is a common practice, due to the increasing awareness on disease prevention. However, consumption of natural antioxidants can be beneficial and may serve as an interesting alternative. Several studies showed that increased dietary intake of natural antioxidant associates with the decreasing of coronary illness (Zadak et al., 2009). According to Ndala et al. (2010), natural antioxidant originated from plant have more advantages in decreasing ROS levels because synergistic

action of wide range of biomolecules such as flavonoids, phenols, phytomicronutrients, vitamin C and vitamin E. Since ubi badak have verified to contain wide range of secondary metabolites, high anthocyanin, antioxidant properties, antimicrobial activities and other medicinal values, it is much suitable to be consume as a healthy food and replace synthetic antioxidant and synthetic food colourant.

However, production of this plant is limited in Malaysia, whereby the tubers can be found only in east cost (Kelantan and Terengganu). The plant also seasonal and tubers are harvested once a year with long dormancy period. Other than supply as a source of food, tuber also act as planting materials. *Dioscorea alata* vegetatively propagated by using tuber pieces. The production of tubers also hampered by several significant virus and fungal diseases. The consumer awareness about this healthy rich food crop also is very low in Malaysia. Therefore, biotechnological methods such as tissue culture techniques will be employed in mass propagated of this food crop, especially to obtain clonal plants with high content of anthocyanins. Plant growth regulators can be manipulated during tissue culture process to mass propagate the plants as well as increase the production of anthocyanin pigments.

Production of coloured callus (containing pigments) will also be achieved via tissue culture techniques and the pigments will be extracted and characterized by means of HPLC and UV-Vis Spectroscopy. Pigments selectively absorb and reflect specific wavelengths of light, thus giving the pigment its distinctive colour (Ashwini & Vinod, 2008). The biosynthesis of these valuable pigments (anthocyanin) is influenced by genetic and environmental factors (Velu & Narayanaswamy, 2009). Therefore, the research aims to evaluate and manipulate the effect of environmental constraints (light intensities and stress conditions) in biosynthesis of anthocyanins. Light has been found to affect the

synthesis of anthocyanin by influencing the level and activity of biosynthetic enzymes (Werner et al., 1998; John & Wesley, 1991). Phenylalanine ammonia lyase (PAL) and the enzymes that convert cinnamate to p-coumaroyl-CoA are rapidly induced under light condition, hence promoting the synthesis of anthocyanins (Giovanni et al., 2011). Stress conditions had also been found to affect pigment biosynthesis (Jesse et al., 2002). Plant growth hormones regulate many important plant growth development processes, also effect the formation of pigment. Changes in the endogenous concentration of the plant growth regulators and the tissue sensitivity in the various plant organs control a wide range of developmental processes. Plant response to plant growth regulators is activated after the binding of the plant growth regulator to a specific receptor in the cell that initiates a cascade of signalling processes.

Human consumes many crop plants as food, therefore increasing the content of valuable pigments in food crops is essential for human health and can help to reduce the dependency on synthetically-produced supplementary health products. *In vitro* technology offers a production alternative of pigments through callus culture, control condition for culture medium composition as well as incubation condition to globalize the production of desired variants with shorter and flexible production cycle. Based on several investigation, compounds produced from an *in vivo* grown plant could be produced at the same or different level or not produced at all in an *in vitro* grown plant. Using these protocol, unlimited planting materials of elite cultivars with high concentration of anthocyanin can be produced, independent of the growing season. Since ubi badak has a good medicinal value but not available throughout the year, *in vitro* (tissue culture) technique can be used to mass propagate this plant. This study also highlighted the importance of *Dioscorea alata* as a healthy food and as a source of natural colourant.

1.2 Research Objectives

1. To establish *in vitro* regeneration system of *Dioscorea alata* L. from leaves, node and stem explants. The effect of plant growth regulators were recorded and the optimum medium for efficient micropropagation, microtuberization, coloured callus induction and somatic embryogenesis of *Dioscorea alata* L. were determined and identified.
2. To investigate the optimum encapsulation matrix for the production of synthetic seeds of *Dioscorea alata* L. from leaves, node and stem explants. The optimum concentration of sodium alginate ($NaC_6H_7O_6$) and calcium chloride dehydrate ($CaCl_2.2H_2O$) were determined.
3. To determine an efficient protocol for acclimatization process of *Dioscorea alata* L. During this period, the plantlets were observed to study the capability of plantlets to adapt to new environments.
4. To determine the optimum light conditions and plant growth regulators combinations and concentrations for over-expression of anthocyanin pigment in *Dioscorea alata* L. grown *in vitro*.
5. To evaluate total phenolic, flavonoids, anthocyanin content and antimicrobial activity of pigments extracts from *Dioscorea alata* L. grown *in vivo* and *in vitro.*

CHAPTER 2: LITERATURE REVIEW

2.1 *Dioscorea alata* L.

Dioscorea alata L. is has the potential to be commercialized as food crop in tropical and subtropical region of the world. This plant has been known to human since ancient times and remain under-utilized. It is native to Southeast Asia such as Malaysia, Indonesia, Philippines, and neighboring countries like Taiwan, Nepal and New Guinea. *Dioscorea alata* was named after Pedianos Dioscorides, a Greek physician from the first century AD whose book on medicinal herbs *Materia Medica*, was a standard work until recent times. Alata means winged in Greek language. *Dioscorea alata* is included in the family of Dioscoreaceae and the genus *Dioscorea*. Generally, the species in the genus *Dioscorea* are dioecious, herbaceous vines and twining with single or clustered large tubers some of which are edible. The large tubers are known as 'yams' (Acevedo-Rodríguez, 2005). The word 'yam' comes from a West African language and means something to eat. Many species of yam are economically important crops worldwide and many of them have been used in the pharmaceutical industry. Only seven species of *Dioscorea* are edible which are *Dioscorea alata* (water yam), *Dioscorea bulbifera* (aerial yam), *Dioscorea cayenensis* (yellow yam), *Dioscorea dumetorum* (bitter yam), *Dioscorea esculenta*, *Dioscorea praehensalis* (bush yam) and *Dioscorea rotundata* Poir (white yam). Varieties of *Dioscorea alata* are the most common yam in the world for cultivation**.** The scientific classification and nomenclature of *Dioscorea alata* is as shown below:

Kingdom : Plantae (Planta, plantae, vegetal, plant)

Subkingdom : Viridiplantae

Infrakingdom : Streptophyta

Superdivision : Embryophyta

Division : Tracheophyta

Subdivision : Spermatophytina

Class : Magnoliopsida

Superorder : Lilianae

Order : Dioscoreales

Family : Dioscoreaceae

Genus : *Dioscorea* L.

Species : *Dioscorea alata* L.

2.1.1 Morphological Description of *Dioscorea alata* L.

Dioscorea alata are herbaceous vine with internodes square in cross section, usually 10 to 15 cm in length and twining to the right. Stems are quadrangular, with four longitudinal winged, undulate, and often tinged in purple colour. Mature stems are cylindrical, spiny, green and reddish in colour. Generally, the leaves of *Dioscorea alata* are large and narrowly heart in shaped. It is located mostly opposite, sometimes alternate on the vine branches. The upper surface of the leaves is shiny and green purplish in colour with venation sunken while the lower surface of the leaves is dull, pale green in colour with promonent venation. The root system for *Dioscorea alata* are fibrous and shallow, for the most part are confined to the top 1 m of the soil.

Dioscorea alata produced two types of tubers, underground tubers and aerial tubers. The underground tubers grow in soil and the aerial tubers formed in leaf axils of the vine. Usually the plant produced only one underground tuber for each season but sometimes up to three. The tubers are varying in shape and size. It is morphologically having rough and bumpy surfaces and large in size. The colour of the tubers are brown with white or cream or purplish (superficially or throughout) pulps. When cultivated, the plant

produced long and uniform tubers. If cultivated in hard soil, the tubers can be lumpy and malformed. Old age tubers also can be lumpy and tend to branch out while young tubers tend to be solid and straight. The tubers utilized best at a young age because older tubers, while still edible would give a different structure. Thus, the older tuber can be lumpy but have a younger, well-formed root attached.

The aerial tubers are produced when the leaves are started to wither. It is pendulous usually located at the branching vine with the size can be reaching up to 15 cm long. The colour and the shape of the aerial tubers produced seen as dark brown in colour and round cylindrical in shape with occasional lumpy morphology being seen.

2.1.2 The Economic Importance of *Dioscorea alata* L.

Dioscorea species was recognized an important tuber crop and is a staple food for millions of people in tropical and subtropical countries before being replaced by more popular cassava plant. Root and tuber crops considered are the most important food crops after cereals. They have the highest rate of dry matter production per day and are major calorie contributors. *Dioscorea* species are recognized as nutritionally more superior compared to other tropical root crops such as cassava and sweet potato. They are a valuable source of carbohydrates, fibers and low level of fats which make them a very good dietary nutrient and also could be processed into various staple intermediate and end product form (Jaleel et al., 2007). *Dioscorea alata* is known for its high carbohydrate content where starch is major carbohydrate reserve accounting for 85% of dry matter and is being studied as an alternative source for starch (Ahmed & Urooj, 2008). The average crude protein of *Dioscorea alata* is 7.4 %, while the vitamin C content ranges from 13 to 24 mg/100g fresh weigh. *Dioscorea* are grown commercially as the main crop in Caribbean Central, South Amerika, West Afrika and India (Tor et al., 1998).

In addition to being an important staple food in many tropical countries, *Dioscorea* receive wide attention due to its functional properties. Dioscorin, the tuber storage protein of *Dioscorea*, and its peptide hydrolysates exhibits angiotensin converting enzyme inhibitory activity (Hou et al., 2000; Hsu et al., 2002). It is suggested that 32 kDa dioscorin, may play a role as antioxidant in tubers and may be beneficial for health when taken as a food additive or when the tubers are consumed. *Dioscorea* is not only enriched in the diet of the people but also possess several medicinal properties to cure many ailments. This plant is popular in Chinese and Indian traditional medicine. Traditionally, the tubers are used to treat appetite disorder, diarrhea, asthma, cough, frequent urination, diabetes and emotional instability. Powdered tubers are prescribed as a remedy for piles, gonorrhea and applied externally to sores.

Dioscorea alata is fast gaining recognition in pharmaceutical industries because of their peculiar of various commercial valuable compounds. *Dioscorea* sp. contain pharmacological active compound, such as diosgenin that is a steroidal type saponin and dioscin, which is a form of diosgenin with sugar attached to it (Ramberg & Nugent, 2002). It is among the ten most important sources of steroids and is also the most often prescribed medicine of plant origin. Diosgenin is a precursor for the chemical synthesis of steroidal drugs, oral contraceptives and is tremendously important to the pharmaceutical industry (Li & Ni, 2011; Kalailingam et al., 2012). Diosgenin plays an important role in the control of cholesterol metabolism, causes variation in the lipoxygenase activity of human erythryoleukaemia cells and is responsible for the morphological and biochemical variation in megakaryocyte cells (Sharareh, 2011). It is reported (Chen et al., 2003) that methyl protodioscin a the potent agent with anti-tumor properties has been synthesis from diosgenin.

Dioscorea alata L. contains high anthocyanin content is correlated with high antioxidant properties (Takamura & Yamagami 1994; Pool-Zobel et al., 1999; Bridle & Timberlake, 1996), antidiabetic (Matsui et al., 2002; Jayaprakasam et al., 2005) improved visual activity (Timberlake & Henry, 1988), antimutagenic (Yoshimoto et al., 1999; Yoshimoto et al., 2001), anticancer activities (Karaivanova et al., 1990; Kamei et al., 1995) and antimicrobial properties.

2.1.3 The limitation of *Dioscorea alata* L.

The biggest limitation in growing *Doscorea alata* is the source of planting materials. *Dioscorea* alata is propagated by using tubers fragments. Besides functioning as the planting materials, tubers also are consumed as the food source. Usually a single plant bears only one tuber or a few and mass propagation through tuber seedling is cost effective. The tubers also effected by viral, fungal and nematodes infection and subsequently lower the tubers yield. Through infected tubers, the defective quality could be transmitted to the next generation and deteriorate the quality of tubers. Viral infection is one of the most serious issue faces by conventional propagation of *Dioscorea alata* L. Once the tubers are infected by a virus, it is transmitted to later generations through tubers and is difficult to be eliminated. Single or multiple virus infection often cause reduction of yield and deterioration of quality of the tubers. The most serious disease that affect *Dioscorea alata* is caused by green-banding virus or mosaic virus. The disease produces symptoms of mosaic which are blotching, crinkling and distortion of leaves and in its extreme phase, reduction of leaf to a thin twister silver. As symptom become severe, the yields of the tubers decrease drastically. Lawson et al. (1974) observed that, aphids might transmit this mosaic virus into sapogenin-bearing yam.

Colletotrichum stoesporioides (fungal) can cause anthracnose disease in *Dioscorea alata*. Anthracnose disease stimulated by the wet condition, especially by dew formation during night time and high temperature and sunlight during the day time. Spores of *C. stoesporioides* are disseminated by wind and by acurvuli on stem and tubers. The spores appear as a black spot that enlarges quickly on leaf and stem followed by yellowing, wilting and leaf loss and in severe cases by death and dieback of the stem. Nematodes especially species of *Prathylenchus*, *Meloidogyne*, and *Scutellonema* the common pest that attack *Dioscorea* tubers. They leave unsightly burrows or swellings that reduce the usability of the tuber tissue.

Severe damage of the tubers can also be caused by several kinds of larvae. *Eteroligmus meles* (Billb.), the greater yam beetle, passes through its mating and reproductive cycle in swampy areas but migrate to yam planting area for feeding. Rotting is closely associated with harvest injury and the main cause of tuber loss in storage. Tubers should not be stored in piles or in damp situations, as these conditions accelerate the rotting process. Rotting is also associated with insect damage and nematode infestation and control of these pests greatly reduces loss in storage.

2.2 Plant tissue culture

Plant tissue culture is a method of *in vitro* cultivation (regeneration) of plant cells and organs, in which the cells divide and regenerate into callus or particular plant organs and subsequently develop into complete plantlet. *In vitro* regeneration or micropropagation of plants refers to multiplication of genetically identical individual (plant) by asexual method of regeneration from organs or somatic embryos. Traditionally, *in vivo* vegetative propagation/ clonal propagation is achieved by cuttings, layering, grafting and budding the vegetative parts. After totipotency of plant cells was established and regeneration of

large number of plants starting from small pieces of somatic tissue could be achieved, tissue culture was projected as potential alternative method for rapid clonal propagation of plants (Murashige, 1974).

Propagation through *in vitro* technique or tissue culture technique is similar to propagation of plant from vegetative part except it was done under sterile condition and the plant part was grow on medium instead of soil. The main aim of *in vitro* regeneration is to produce a lot plantlets, genetically and physiologically uniform with high photosynthetic potential and able to survive the *ex vitro* environment (Jeong et al., 1993; Solarova & Pospisilova, 1997). *In vitro* regeneration has numerous advantages compare to conventional proliferation method. The several advantages were listed as below:

i) Mass propagation of plant can be initiated from only a small tissue from one individual.
ii) Very small part of plant tissue needed to initiate aseptic culture.
iii) The frequency of multiplication *in vitro* is often much faster than the in vivo methods.
iv) Plant can be produced all year round not depending on season and environmental factor.

2.2.1 Organogenic Micropropagation

In vitro regeneration or micropropagation via organogenesis in plant is a process where formation and multiplication of organs (microshoots and root) which is genetically identical to the mother plant from somatic tissue by asexual method. Organogenesis is the process by which cell and tissue are forced to undergo changes that could leads to the production of a unipolar structure namely a shoot or root primordium, while vascular system is often connected to the parent tissue (Thorpe, 1998; Victor et al., 1999). In early

1960s, when totipotency of plant cell as established and regeneration of large number of plants starting from small pieces of somatic tissues could be achieved, tissue culture was projected as a potential alternative method for rapid clonal propagation of plants (Murashige, 1974).

The development of organs (shoots, roots or flowers) and overall plant shape and structure are known as morphogenesis. *In vitro* plant morphogenesis can be accomplished through organogenesis and somatic embryogenesis pathway. According to De Klerk (2003), differentiated somatic cells can initiated the ontogenic programme. When the somatic cell was treated with the right stimuli, they would develop into an adventitious meristem. From the adventitious meristem, adventitious shoots and embryo will be generated. The development of meristem shoots is mentioned as organogenesis and development of embryo is alluded as somatic embryogenesis. Victor et al. (1999) and Thorpe (1980) indicated that organogenesis is the forced changes process of cells and tissue to produce shoot or root primordium which vascular system attached to the parent tissue. Three phases of organogenesis were identified as below;

i) Cells and tissue (explants) acquire organogenic competence which is described as the capability to react to hormonal signal of organ induction (Sugiyama, 1999).

ii) The competence cells or tissue are guided and determined for specific organ formation under influence or effect of plant growth regulators (plant growth regulators).

iii) Organogenesis continues independently of the supplied plant growth regulators.

Organogenesis occur either directly or indirectly. Through direct organogenesis, development of plant organs such as shoots, roots, microtuber etc. arise directly from the tissue cultured. While, for indirect organogenesis, explant cultured undergo callus phase

before developing into plant organs. Callus is defined as undifferentiated cells usually develop on wound of differentiated tissue. According to Halperrin (1969) callus usually composed of unspecialized parenchyma cells. Formation of callus can be induced from various plant parts such as leaves, node, stem, petiole, root etc. through *in vitro* system. Exogenous plant growth regulators or hormones are essential to initiate callus formation. The type and concentration of plant growth regulators required are varied depends on the explant type and plant species. Usually to prompt the callus formation, auxin and cytokinins are included in the callus induction medium. According to Rayle et al. (1992), auxin play a significant role in cell elongation, division and differentiation. While, Dietz et al. (1990) mention that, the function of cytokinin are to enhance the enlargement of plant cells. Production of callus can be enhance using suspension culture or liquid culture. Suspension cultures offer a system for fast growth and development of plants. Razdan (1993) stated that cells in suspension cultures obtain more homogeneous stimuli from the medium fortified with the necessary amount of inducer such as plant growth regulators and sucrose.

Microtubers is an alternative end product of micropropagation produced by allowing *in vitro* plantlets to grow under tuber inducing conditions. Microtubers have become an important starting material for rapid multiplication as well as germplasm storage and transfer. Besides the other parts of plants, microtubers can also be utilized as an explant for *in vitro* regeneration.

Skoog & Miller (1957) proposed the concepts of hormonal control in organogenesis. In their research, the formation of organ (bud and root) were controlled by auxin and cytokinins level. They found out auxin in high concentration enhanced the formation of roots while high cytokinins concentration promote bud and shoot formation. According

to Evans et al. (1981), interaction of auxin and cytokinins are normally viewed as the most significant for growth regulation and development in plant tissue cultures as these two types of plant growth regulators are commonly necessary for morphogenesis and organogenesis.

2.2.2 Embryogenic Micropropagation

Regeneration of complete plantlets can also be achieved via somatic embryogenesis pathway other than organogenesis pathway. Somatic embryogenesis is the regeneration process of somatic cells initiating the developmental pathway to form complete embryos known as somatic embryos. Somatic embryos have the similar ability as zygotic embryos which is to regenerate complete plantlet. Somatic embryogenesis occurs through the same key stages of embryo development as zygotic embryogenesis namely globular, heart shape and torpedo stages in dicots. The zygotic embryo in monocots are more complex as compared to dicots cause different of key stages of somatic embryos were observed namely globular, scutellar (trasition) and coleoptilar stages. Somatic embryogenesis was first induced in suspension culture (Stewart et al., 1958) and in callus culture (Reinert, 1959) of carrot.

There are two types of somatic embryogenesis, direct somatic embryogenesis and indirect somatic embryogenesis. Direct somatic embryogenesis occurred when, somatic embryos was directly induced from already pre-embryogenic cells such as leaves, node or microspores without the formation of callus. Cells that are capable of direct somatic embryogenesis are physiologically similar to those in zygotic embryos. Indirect somatic embryogenesis occurs when callus produced leads to the formation of somatic embryos. Somatic embryogenesis occurred either directly or indirect should be considered as two extremes of a continuum (Carman, 1990; Wann, 1988; William & Maheswaran, 1986). It

is not always clear which types occurs or both direct and indirect can be observed. According to Reamakers et al. (1995), if the period of somatic embryogenesis is short the process will be direct somatic embryogenesis but if the process takes longer than the process will be indirect somatic embryogenesis.

Somatic embryogenesis is influence by the presence of plant growth regulators in the culture medium. The induction of somatic embryogenesis consists of the termination of the existing gene expression pattern in explant tissue, and its replacement with an embryogenic gene expression program in those cells of the explant tissue which will give rise to somatic embryogenesis (Merkle et al., 1995). A treatment with the ability to down regulate gene expression can stimulate somatic embryogenesis. One possible mechanism to down regulate gene expression is DNA methylation, which has been found in study to correlate with the amount of exogenous auxin. 2,4-D was the most commonly applied auxin for somatic embryogenesis induction. Cytokinins usually shows negative effect when applied in the somatic embryogenesis induction medium. Ling et al. (1989) and Ranga Swamy (1961) shows that high level of cytokinins in leaves associated with deficiency embryogenic response in napier and orchard grass. Kachba et al. (1972); Raj Bhansali & Arya (1977) and Dhillon et al. (1989) found that the percentage embryogenesis was decreased when a cytokinin is used in combination with auxin.

2.2.3 Synthetic Seed Production

Synthetic seed are defined as artificially encapsulated somatic embryos, shoots or other tissues which can be used for sowing under *in vivo* or *ex vitro* conditions that are able to grow into plantlets after sowing (Bapat & Rao, 1990; Aitken-Christie et al., 1995). Synthetic seed are considered as the most effective and efficient alternative methods for *in vitro* propagation of plant species that have problems in seed propagation and for plants

which produced non-viable seeds or without seeds. Its potential advantages include stabilities during handling, potential for long term storage without losing viability, transportation and planting directly from *in vitro* to field conditions and higher scale at a low-cost production (Ghosh & Sen, 1994).

Dioscorea alata do not produce natural seeds. Traditionally, this plant is propagated by using tuber segments. However, there are several limitations by using tubers as planting materials. Moreover, tuber is also the main source of food materials in *Dioscorea alata*, by using tubers as the planting materials, this would introduce competition for the usage of plant parts. Hence, the development of synthetic seeds of *Dioscorea alata* as an alternative or substitute planting materials is crucial. Therefore, the objective of the present study was to develop an alternative propagation method for *Dioscorea alata* via synthetic seed production.

2.2.4 Acclimatization

Acclimatization is an adaptation process of *in vitro* grown plantlets to the natural environment or *ex vitro* environment. Pospisilova et al. (1999) and Hazarika (2003) stated that, *in vitro* acclimatization is one of the key factors in producing healthy plantlets before they are transplanted to *ex vitro* conditions. Micropropagation is considered successful only when plantlets after transfer from *in vitro* culture to the soil showed high survival rates. Usually high percentage of plantlets cannot survive the *ex vitro* environment due to extremely different condition between *in vitro* and *ex vitro*. Plantlets grown in *in vitro* environment are not fully developed within the culture containers with high air humidity under aseptic conditions, low level of light, on a medium containing ample sugar and nutrients to allow for heterotrophic growth and in an atmosphere with high level of humidity. Low gas exchange in the culture container, CO_2 shortage and relatively low

photosynthetic photon flux density (PFD), induces disturbance in plant development and photosynthetic performance in *in vitro* plantlets (Kozai, 1991; Pospisilova et al., 1997l).

The physiological and anatomical characteristics of *in vitro* grown plantlets should be gradually acclimatized to the natural environment. Therefore, in this study, plantlets from tissue culture containers were transferred step by step to the green house (natural environment) to observe the adaptation capability of the plantlets. The aim of this study was to measure the survival rate of acclimatized plantlets of *Dioscorea alata*. The success of the plantlets to adapt to the *ex vitro* condition means the successful of tissue culture protocol of this plant. Soil content of the sowing media will be analyzed for their texture and fertility content. The suitable sowing media was selected for plantlet acclimatization.

2.2.5 Micropropagation of *Dioscorea alata* L.

In agriculture or in the conventional propagation of *Dioscorea alata*, tubers are utilized as the source for plantings materials. Tuber segment known as 'tuber seeds' are grown and propagated to form into another tuber. The absence of viable seeds, the long period required for obtaining usable tubers and phytosanitary problems are some factors limit the rapid conventional propagation and economic exploitation of *Dioscorea* species (Bolagum et al., 2006; Tschannen et al., 2005). As many species of *Dioscorea*, *Diocorea alata* is dioecious and cultivated form have the larger diversity compared to the cultivated forms of other species (Mantel et al., 1978). It is then necessary to propagate vegetatively the selected plant of this species.

Plantlet regeneration *in vitro* for vegetative propagation of some economically important *Dioscorea* species has been reported previously using nodal vine cuttings (Yan et al., 2002; Chen et al., 2003), zygotic embryo (Viana & Mantel, 1989), meristem tips

(Malaurie et al., 1995a; Malaurie et al., 1995b), bulbils (Asokan et al., 1983), immature leaves (Kohmura et al., 1995), root (Twyford & Mntell, 1996) cell and protoplasts (Tor et al., 1998). Much attention has been paid to the clonal propagation through *in vitro* production of microtubers in *Dioscorea abyssinica* (Martine & Cappadocia, 1991), *Dioscorea batatas* (Koda & Kikuta, 1991), *Dioscorea composite* (Alizadegh et al., 1998), *Dioscorea floribunda* (Sengupta et al., 1984) and *Dioscorea alata* (Jasik & Mantell, 2000). Though, only few studies have been reported on the microprogation and microtuberization of *Dioscorea alata*.

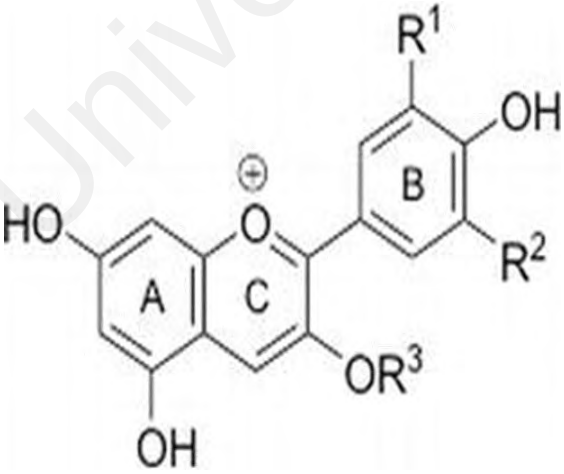
In general, various results achieved from micropropagation of *Dioscorea* shows that it is possible to mass propagate *Dioscorea alata in vitro* from different explants such as node, leaves fragment, stem and microtubers. *In vitro* method of vegetative multiplication of *Dioscorea alata* would have considerable benefits for the medicinal trade and germplasm conservation. This alternative and application of plant tissue culture offers valuable ways to overcome all the problems that are found in natural propagation. *In vitro* propagation of *Dioscorea alata* is more efficient than conventional propagation and definitely helpful for obtaining a large-scale diseases free seedling and ensure adequate supply of quality plantlets to meet different purpose and conservation.

2.2.6 Plant Tissue Culture for Production of Antimicrobial Phytochemicals

Plant secondary metabolism produce most valuable phytochemicals (Nehra, 1994). Due to their large biological activities, plant secondary metabolites have been used for centuries in traditional medicine. According to Tripathi & Tripathi (2003), World Health Organization estimated that up to 80% of people in the world were still rely mainly on traditional medications such as herbs for their illness treatment. Tripathi & Tripathi (2003) also mention that, it is estimated that approximately one quarter of prescribed

drugs modern drugs contain plants extracts or active ingredients obtained from or modeled on plant substances. Survey conducted in western countries, where chemistry is the backbone of the pharmaceutical industry shows that 25% of the molecules used for drugs manufacturing were originated from natural plant (Payne et al., 1991). The most popular analgesic, aspirin was originally derived from species of *Salix* and *Spiraea* and some of the most valuable anti-cancer agents such as paclitaxel and viblastine are derived solely from plant source (Katzung, 1995; Taxol, 1996; Roberts, 1988).

Plant secondary metabolites usually classified according to their biosynthetic pathway (Harborne, 1999). Three large molecule families are generally considered: phenolics, terpenes and steroids and alkaloids (Bourgaud et al., 2001). Phenolic compounds are widespread metabolite family in plants. Anthocyanins is plant-derived flavonoid that are responsible for red, purple and blue hues present in fruits, vegetables and grains. They are present in a wide range of plant tissues, principally flowers and fruits, but also storage organs, roots, tubers and stems. The basic structure of anthocyanins are based on C_6-C_3-C_6 skeleton of flavonoids which consist of 15 carbon atoms.



	R_1	R_2
Cyanidin	OH	H
Delphinidin	OH	OH
Malvidin	OCH_3	OCH_3
Pelargonidin	H	H
Peonidin	OCH_3	H
Petunidin	OCH_3	OH

Figure 2.1: Structure of the most common anthocyanidins occurring in nature.

Anthocyanidin or aglycone is the main part of anthocyanins. Anthocyanidins consist of an aromatic ring A bonded to an heterocyclic ring C that contains oxygen, which is also bonded by a carbon-carbon bond to aromatic ring B (Figure 2.1). The conjugated double bonds in the anthocyanidin structure is responsible for absorption of light around 500nm causing the pigments to appear red to human eye. There is a huge variety of anthocyanins spread in nature, however the most commonly found anthocyanins are cyaniding, delphinidin, malvidin, pelargonidin, peonidin and petunidin. The main differences between them are the number of hydroxyl and methoxyl groups in the B ring of the flavylium cation.

Early industrial use of anthocyanin pigment was for fabric dyes. Recently, anthocyanin is widely acknowledged as having significant health-giving properties such as antioxidant, anti-inflammatory, anti-ulcer and wound healing properties (Lila, 2004). Research has also shown that many artificial pigments are actually detrimental to our health and natural antioxidant are high in demand because of their potential in health promotion and disease prevention and their improved safety and consumer acceptability. With an increasing consumer preference for healthy foods, there is now considerable demand for the use of anthocyanins as the natural food colourants, due to their natural pedigree and healthful properties.

The past three decades has seen a dramatic increase in microbial resistance to antimicrobial agents that lead to repeated use of antibiotics and insufficient control of the disease (NCID, 2002). The terms of antimicrobials agents include antibacterial, antifungal, antiprotozoal, antihelminthic and antiviral agents. The majority of clinically used antimicrobials have various drawback in terms of toxicity, efficacy, cost, and their frequent use has led to the emergence of resistant strain. Misuse, abuse and over-

prescription of antibiotics are believed to be the reasons of emergence of resistance (Eloff, 2000; Peterson & Dalhoff, 2004). Hence, there is a great demand for a new source of antimicrobial agents selectively acting on new targets with fewer side effects. One approach might be the testing of plant traditionally used for their antimicrobial properties as potential source for drug development.

Traditional therapeutic value of plants to treat common microbial infection diseases has long been recognized in many different communities and some of these traditional medicines are still included as part of the habitual treatment of various illnesses. Several plant species are used by many tribal groups for the treatments of various ailments ranging from minor infections to dysentery, skin diseases, athma, malaria and a horde of other indication (Perumal Samy & Ignachimuthu, 2000; Dahanukar et al., 2000). Furthermore, it is estimated that two-thirds of the world population rely on traditional remedies due to the limited availability and high prices of most pharmaceutical products (Tagboto & Towson, 2001).

One alternative source for the production of anthocyanins and antimicrobial is through the utilizing of plant cell cultures (Delgado-Vargas et al., 2000; Ramachandra & Ravishankar 2002; Lila 2004). Plant secondary metabolites can be producing *in vitro* via plant cell culture (Barzn & Ellis, 1981; Deus & Zenk, 1982). The use of plant cells and tissue culture methodology as means of producing medicinal metabolites has a long history (Rout et al., 2000; Verpoorte et al., 2002). Plant tissue culture is an alternative method for commercial propagation of plants and is being used widely for the commercial propagation of large number of plant species including many medicinal plants. Cultured plant cells can also synthesize, accumulate and sometime exude many classes of metabolites (Matkowski, 2008).

2.2.7 Factors Influencing the Success of Tissue Culture

The successful of plant tissue culture depends on the composition of the culture medium. Generally, plant tissue culture medium comprised carbon source, organic compound, inorganic compound (consist of macro and micro nutrients), solidifying agent and plant growth regulators. Sugar usually sucrose was normally added into the culture medium as a carbon source or energy source. In tissue culture photosynthesis is insufficient because the condition in the culture container unsuitable for photosynthesis or without photosynthesis. Due to this reason, additional of sugar in the culture medium are crucial factor, to supply energy for *in vitro* initiation, growth and development. Additional of sugar also help to maintain the osmolarity of the culture medium. Pierik (1987), observed that, high sugar concentration is needed in the culture medium for adventitious root formation, especially in the case of woody plants.

Plant growth regulators (hormones) also play an important role in the successfulness of *in vitro* plant tissue culture. Plant growth regulators are organic compounds naturally synthesized in higher plants, which can influence the growth and development of plant tissue. Artificial or synthetic plant growth regulators have been developed which correspond to the natural ones. Auxins and cytokinins are the more important plant growth regulators use in tissue culture. The discoveries of auxin and cytokinins as well as their functions, are finally revealed that shoot and root regeneration was regulated by plant growth regulators in tissue culture system. There are various kinds of auxins: 1 naphthaleneacetic acids (NAA), 1H-indole-acetic acid (IAA), 1H-indole-3-butyric acid (IBA) and 2,4-dichlorophenoxyacetic acid (2,4-D). Commonly, auxin was supplemented into the culture medium for root induction and development. Auxins usually cause cell elongation, cell division, swelling of tissues, the formation of adventitious roots and the inhibition of adventitious and axillary shoots formation, often embryogenesis. In several

cases additional of high concentration of auxin, is inhibitory for root formation. Additional of high auxin the media can induce the formation callus. Pierik (1987) mention that, auxin at low concentrations, was predominated for ventitious roots formation, whereas high auxin concentrations root formation fails to occur, and callus formation will take place.

The commonly used synthetic auxin in tissue culture are IAA, IBA (tend to denature in media and rapidly metabolized within plant tissue), 2,4-D (often used for callus induction and suspension culture), and NAA (when organogenesis is required). Among others, dicamba (3,6-dichloro-*o*-asinic acid) and picloram (4-amino-3,5,6-trichloropyridine-2-carboxylic acid) are often effective in inducing the formation of embryogenic tissue or in maintaining suspension cultures (Gray & Conger, 1985; Hagen et al., 1991). BSAA (benzo(b)selenienyl-3-) acetic acid) is another synthetic auxin with powerful auxin like activities (Lamproye et al., 1990; Gasper, 1995).

Cytokinins are plant growth regulator derivative from adenine. Cytokinins often used to induce cell division, modification of apical dominance and shoots dedifferentiation in tissue culture. The most commonly used cytokinins in tissue culture are the substituted purines, BAP (6-benzylaminopurine) or BA (6-benzyladenine) and kinetin (N-1-H-purine-6-amine). Adenine, adenosine and adenylic acid may have the cytokinin activity although less than that of the cytokinins (Gaspar et al., 1996). Adenine can be used to bring about or reinforce response normally attributed to cytokinin action.

The interaction between auxin and cytokinin is important with respect to morphogenesis in tissue culture system. According to Razdan (1993), for somatic embryogenesis formation, callus initiation and root initiation the requisite ratio of auxins

to cytokinin is high while the reverse leads to axillary and shoot proliferation. Additional of auxin in higher concentration (1-10 mg/l) can induce adventitious shoot formation and root formation is generally inhibited whereas additional higher concentration of cytokinins can induce shoot formation thus inhibited root formation (Pierik, 1987).

Macronutrients and micronutrients are important component for tissue culture media. Macronutrient are mineral elements that required by plants in concentration which is greater than 0.5 mmol l^{-1} (Appendix A). Macronutrients are essential components of macromolecules such as protein and nucleic acids as well as constituents of many small molecules (Carl & Richard, 2002). Nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), Magnesium (Mg) and Sulphur (S) are the major element in macronutrient. Micronutrients are required in much smaller quantities less than 0.005 mmol l^{-1} and essential in various roles such as enzymes cofactors or components of electron transport protein (Marschner, 1995). Micronutrients consist of iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu), molybdenum (Mo), cobalt (Co), iodine (I) and sodium (Na).

Organic nutrients that usually added into the media are amino acids, activated charcoal, antibiotics, myo-inositol, vitamins and other organic supplements like protein (casein) hydrolysates, coconut milk, yeast and malt extracts, ground banana, orange juice and tomato juice. Vitamins are supplemented in small quantities into the tissue culture media to induce the best growth of the tissue. Thiamin (B_1), Nicotinic acid (B_3), and Pyridoxine (B_6) are the commonly vitamin used in plant tissue culture. Razdan (1993) reveal that, Thiamine is the basic vitamin required by all plant cells and tissues. Most plants are able to synthesize vitamins *in vitro*. Amino acid also important in tissue culture improvement by stimulating the cells growth. Additional of amino acids in the culture media proven to inhibit the cell growth while their mixtures are frequently beneficial (Razdan, 1993).

Medium fortified with adenine generally can enhanced the cells growth and adventitious shoot formation. Adenine was first used by Skoog & Tsui (1948) in the tissue culture of tobacco.

Myo-inositol is a sugar like carbohydrate or sugar alcohol produced by most plants. Myo-inositol is a staple ingredient of media used to grow plant tissue though the role of myo-inositol in the successful growth of the tissues culture has not been determined. Myo-inositol is the essential molecule for the production of the plant cell wall. It is supplemented component in various root products because it is significant function for phosphate storage, plant growth regulators storage and transport, cell wall biosynthesis and the production of stress related molecules. An oxidize form of inositol is the most common and important sugar involved in polysaccharide production for cell walls (Loewus & Murthy, 2000). Bandurski (1979) mention that, myo-inositol has been assumed to play a vital role in the control of auxin transportation. It links up with auxin to form inactive hormone-conjugates that allowed for safe auxin storage and transport and may regulate the available of active auxin for physiological responses. Sometimes activated charcoal was added in tissue culture media to stimulate growth and differentiation of plant cells. Studies were reported on orchids, tomato, ivy and carrot. Activated charcoal also added in the tissue culture medium helps to reduce toxicity by removing toxic compounds like phenolic compounds produced during the culture and permits unhindered cell growth.

Environmental factors have great influence in the process of differentiation and growth of tissues in cultures system. Plant cells and tissues usually need optimum pH for growth and development. The pH medium can affect the ions uptake of the cell. In general, the pH reading that is appropriate for most plant tissue and cells is around 5.00-6.00. pH value

can influence the solidifying process the tissue culture medium. pH reading higher than 6.00 usually produce hard medium while a low pH result in unsatisfactory solidification of the agar. Culture room also play significant role in the successful of *in vitro* tissue culture. The cultures must be kept or maintained in a space which light, temperature, air circulation and humidity that can be controlled. It is very difficult to designate whether a particular culture should be grown in the light or the dark, at high or low temperature but the best to choose is those light and temperature conditions that are the best for the growth and development of the experimental materials *in vivo* Peirik (1987). According to Razdan (1993), a diurnal illumination of 16 hours day and 8 hours night is generally found satisfactory for multiplication and proliferation of shoots for most plant cells and tissue. The temperature in the culture room usually maintain at 25ºC. The optimal temperature for *in vitro* growth and development is generally 3-4 ºC higher than *in vivo* growth (Peirik, 1987). The volume of the culture container also can affect the cells and tissue growth. The volume of the culture container can affect the constitution of the gas phase within the culture vessel. Ethylene, oxygen, carbon dioxide, ethanol and acetaldehyde are metabolically active gases with possible effects on morphogenesis and may promote unorganized cell growth (Razdan, 1993).

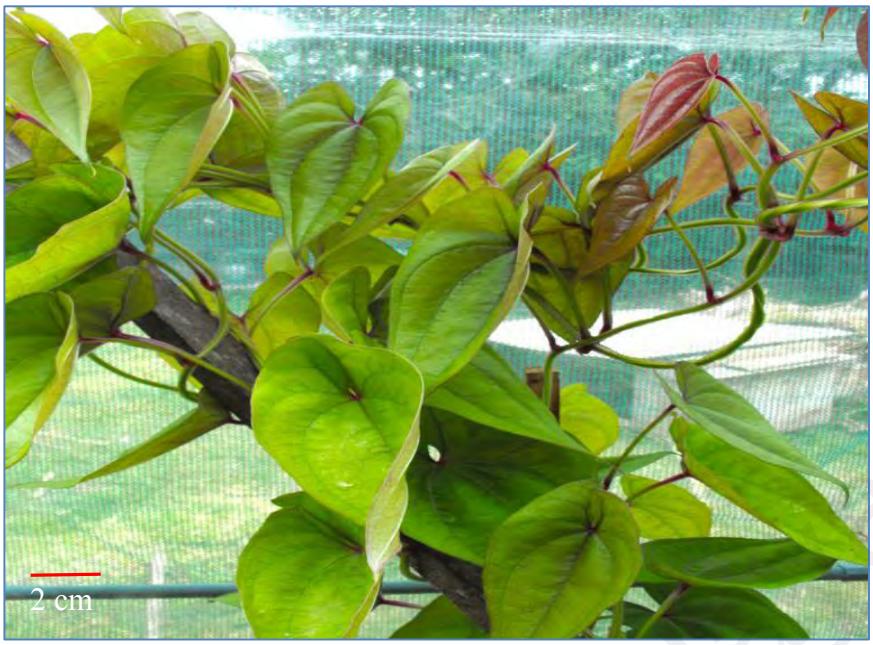


Figure 2.2: Intact plant of *Dioscorea alata* L. grown at Universiti Malaya green house.

Figure 2.3: Purple-tinged stem and narrowly heart shape leaves of *Dioscorea alata* L.

Figure 2.4: Irregular shape tubers with rough and bumpy surfaces.

Figure 2.5: Tubers with intense purple colour.

CHAPTER 3: METHODOLOGY

3.1 Regeneration of *Dioscorea alata* L. *in vitro*

3.1.1 Explant Preparation

3.1.1.1Plant Materials

Tubers of *Dioscorea alata* L. were collected from local grower in Machang, Kelantan, Malaysia and germinated at Institute of Biological Sciences, Faculty of Science, Universiti Malaya, Kuala Lumpur, Malaysia. The tuber was cut into 8 cm segments with peel and small parts of flesh then cultured in plastic pots containing garden soil. After 3 months, each apex had grown into a long stem with 10 to 15 nodes. Leaf, stem (internode) and node obtained from this plant were utilized as explants for *in vitro* cultures.

3.1.1.2 Sterilization of explants

Explants (leaf, stem and node) from 3 month-old intact plants of *Dioscorea alata* were utilized as explants for *in vitro* cultures. First, explants were washed with Teepol and antiseptic germicide Dettol and surface sterilized under running tap water for 30 minutes to remove contaminants and any residues that found on the explants. After that, the explants were soaked in different concentrations (70%. 50%, and 30%) of sodium hypochlorite (clorox) solution. At the first soaked of 70% sodium hypoclorite, two drops of Tween 20 was added. The explants were then rinsed with sterile distilled water to get rid of any traces of sodium hypochlorite that was used earlier. Each rinse lasted approximately for three minutes. Finally, in the laminar flow chamber, the explants were soaked in ethanol 70% for three minutes then washed 3 times in sterile distilled water for three minutes prior to culturing.

3.1.2 Preparation of Culture Media

In this study, MS basal media and MS media fortified with plant growth regulators (BAP and NAA) were prepared. MS (Murashige & Skoog, 1962) was used as the basic medium. MS media powder was purchased from Sigma Chemical Company, Sigma-addrich. To prepare 1 litre MS basal media, 1000ml conical flask were filled up with 800ml of distilled water. After that, 4.4 g MS powder and 30 g sucrose, were weighed and added into the conical flask. The conical flask was placed on the hot plate and the medium solution was stirred until all the sucrose and MS powder were dissolved. After that, distilled water was added, made up to 1 litre in the conical flask. The pH of the medium was adjusted to 5.8 using 1.0 M hydrochloric acid (HCl) and 1.0 M sodium hydroxide (NaOH). Next, 3.5 g gelrite gellan gum was added into the medium. The media was autoclaved at a pressure of 104 kPa (15 Psi^2) and temperature of 121ºC for 20 minutes. For MS medium supplemented with plant growth regulators, stock of plant growth regulators was prepared by diluting the plant growth regulators in distilled water. First, to prepare 1 mg/l stock plant growth regulators, 100 mg of plant growth regulators was weighed and dissolved in a few drops of HCl or NaOH. Then, 100 ml of distilled water was added into the solution. The mixture was stirred until clear solution was obtained. Plant growth regulators was added into the medium prior to autoclave. After the media has been autoclaved and cooled (50ºC), the media were dispensed into 60 ml sterile universal container.

3.1.3 Culture Conditions

Prior to tissue culture work, all equipment and apparatus essential for culture must be sterilized. All tissue culture apparatus like forceps, scalpels, jam jars and etc.were autoclaved before used. Blades, universal containers and Petri dishes were sterile before used. The laminar flow chamber, in which all tissue culture work was done, was sterilized

by spraying all the surfaces with 70% ethanol and then wiped with autoclaved tissues. Before that, the shortwave length ultraviolet (UV) lamp was switched on in the chamber for about 15 minutes. All apparatus and related tools must be wiped with 70% ethanol prior to culturing. Scalpels and forceps were dipped into hot bead sterilizer and cooled in sterile distilled water before used to excise the explants. The hot bead sterilizer was switch on for about 15 minutes before used (to get the desirable temperature of 250ºC).

3.1.4 Identification of Suitable Plant growth regulators and Explants for *In vitro* Regeneration

The effect of MS basal media and MS media fortified with BAP and NAA applied singly or in combination were studied to obtain the optimum medium for microshoots and root formation. Leaf, stem (internode) and node were utilized as the explant. Leaf explants were cut into segments (1 cm x 1 cm), stem explants were cut into 1 cm in length and node explants were isolated from stem were cultured on media prepared. All explants cultured were maintaned in the culture room at 25±1 ºC and 16 hours light of photoperiod with 25µmol $m^{-2}s^{-2}$ of light intensity for 16 weeks. Results were recorded during 16^{th} week. From the results obtained in this experiment the most responsive explant and the optimum medium for root regeneration were recognized.

3.1.4.1 Effect of BAP and NAA Applied Singly or in Combination on Microshoots Formation

All concentrations and combination of plant growth regulators were tested for microshoot formation from leaf, stem and node explants of *Dioscorea alata* were as listed below;

1. MS media (as control)
2. MS + 0.5 mg/l NAA

3. MS + 1.0 mg/l NAA

4. MS + 1.5 mg/l NAA

5. MS + 2.0 mg/l NAA

6. MS + 2.5 mg/l NAA

7. MS + 3.0 mg/l NAA

8. MS + 0.5 mg/l BAP

9. MS + 1.0 mg/l BAP

10. MS + 1.5 mg/l BAP

11. MS + 2.0 mg/l BAP

12. MS + 2.5 mg/l BAP

13. MS + 3.0 mg/l BAP

14. MS + 0.5 mg/l NAA+ 0.5 mg/l BAP

15. MS + 0.5 mg/l NAA+ 1.0 mg/l BAP

16. MS + 0.5 mg/l NAA+ 1.5 mg/l BAP

17. MS + 0.5 mg/l NAA+ 2.0 mg/l BAP

18. MS +0.5 mg/l NAA+ 3.0 mg/l BAP

19. MS + 1.0 mg/l NAA+ 0.5 mg/l BAP

20. MS + 1.0 mg/l NAA+ 1.0 mg/l BAP

21. MS + 1.0 mg/l NAA+ 1.5 mg/l BAP

22. MS + 1.0 mg/l NAA+ 2.0 mg/l BAP

23. MS + 1.0 mg/l NAA+ 3.0 mg/l BAP

24. MS + 1.5 mg/l NAA+ 0.5 mg/l BAP

25. MS + 1.5 mg/l NAA+ 1.0 mg/l BAP

26. MS + 1.5 mg/l NAA+ 1.5 mg/l BAP

27. MS + 1.5 mg/l NAA+ 2.0 mg/l BAP

28. MS + 1.5 mg/l NAA+ 3.0 mg/l BAP

29. MS + 2.0 mg/l NAA+ 0.5 mg/l BAP

30. MS + 2.0 mg/l NAA+ 1.0 mg/l BAP

31. MS + 2.0 mg/l NAA+ 1.5 mg/l BAP

32. MS + 2.0 mg/l NAA+ 2.0 mg/l BAP

33. MS + 2.0 mg/l NAA+ 3.0 mg/l BAP

34. MS + 3.0 mg/l NAA+ 0.5 mg/l BAP

35. MS + 3.0 mg/l NAA+ 1.0 mg/l BAP

36. MS + 3.0 mg/l NAA+ 1.5 mg/l BAP

37. MS + 3.0 mg/l NAA+ 2.0 mg/l BAP

38. MS + 3.0 mg/l NAA+ 3.0 mg/l BAP

3.1.4.2 Identification of Suitable Plant growth regulators and Explants for Root Regeneration

Several concentrations and combination of plant growth regulators were tested for root formation from leaf, stem and node explants of *Dioscorea alata*. MS free plant growth regulators was used as a control. The plant growth regulators concentrations and combinations tested were as listed below;

1. MS media (as control)

2. MS + 0.5 mg/l NAA

3. MS + 1.0 mg/l NAA

4. MS + 1.5 mg/l NAA

5. MS + 2.0 mg/l NAA

8. MS + 0.5 mg/l BAP

9. MS + 1.0 mg/l BAP

10. MS + 1.5 mg/l BAP

11. MS + 2.0 mg/l BAP

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

3.1.5 Induction and Production of Microtubers

3.1.5.1 Identification of Suitable Plant growth regulators for Microtubers Formation

Four month-old plantlets derived from direct regeneration of node explants were subcultured onto MS media supplemented with different concentrations and combinations of plant growth regulators for microtubers formation. Four concentrations of BAP, eight concentrations of Kinetin and four concentrations and combination of BAP and NAA were prepared. The following is the list of media that were used in this experiment.

1. MS basal (medium without plant growth regulators/ control)

2. MS + 0.5 mg/l BAP

3. MS + 1.0 mg/l BAP

4. MS + 1.5 mg/l BAP

5. MS + 2.0 mg/l BAP

6. MS + 0.5 mg/l Kin

7. MS + 1.0 mg/l Kin

8. MS + 1.5 mg/l Kin

9. MS + 2.0 mg/l Kin

10. MS + 1.0 mg/l BAP + 0.5 mg/l NAA

11. MS + 1.0 mg/l BAP + 1.0 mg/l NAA

12. MS + 1.0 mg/l BAP + 1.5 mg/l NAA

13. MS + 1.0 mg/l BAP + 2.0 mg/l NAA

All plantlets were maintained in the culture room at 25±1 ºC and 16 hours light of photoperiod with 25µmol $m^{-2}s^{-2}$ of light intensity for 16 weeks. Observation were made every month and results were recorded during the 10^{th} month. From the results obtained in this experiment, the optimum medium for microtubers formation was identified.

3.1.5.2 Identification of Suitable Plant growth regulators for Shoot Proliferation from Microtuber

Each microtuber produced was cut into small segments (1 cm) and cultured on MS medium supplemented with 30 g/l sucrose and 3.5 g/l gelrite gellan gum. MS culture medium was used together with different types, concentrations and combinations of plant growth regulators. The following is the list of media that were used in this experiment;

1. MS basal (as control)

2. MS + 0.5 mg/l BAP

3. MS+1.0 mg/l BAP

4. MS+1.5 mg/l BAP

5. MS+2.0 mg/l BAP

6. MS+0.5 mg/l NAA

7. MS+1.0 mg/l NAA

8. MS+1.5 mg/l NAA

9. MS+2.0 mg/l NAA

10. MS + 1.0 mg/l BAP + 0.5 mg/l NAA

11. MS + 1.0 mg/l BAP + 1.0 mg/l NAA

12. MS + 1.0 mg/l BAP + 1.5 mg/l NAA

13. MS + 1.0 mg/l BAP + 2.0 mg/l NAA

14. MS+2.0 mg/l NAA +0.5 mg/l BAP

15. MS+2.0 mg/l NAA +1.5 mg/l BAP

16. MS+2.0 mg/l NAA +2.0 mg/l BAP

All microtubers cultured were incubated in the culture room at 25±1 ºC with 16 hours light and 25μmol $m^{-2}s^{-2}$ of light intensity for 16 weeks. Observations were made every weeks and results were recorded during the 3rd month. From the results obtained in this experiment, the percentage of explants produced shoots and the average number of shoots were evaluated and the optimum medium for shoot formation from microtuber was identified.

3.2 Coloured Callus Induction from Various Explants of *Dioscorea alata* L.

3.2.1 Explants Preparations

Leaf, stem (internode) and node explants obtained from 6-month-old plantlets were used in this experiment. Plantlets were induced from nodes of intact plants cultured on MS basal media supplemented with 30 g/l sucrose and 3.5 g/l gelrite gellan gum (as discussed in section 3.1.4).

3.2.2 Preparation of Culture Media

Media was prepared using the same steps discussed in section 3.1.2. MS was used as the basic medium for callus induction. Desired plant growth regulators, myo-inositol and activated charcoal was added into the medium. Same steps was followed for liquid media preparation except no gelrite gellan gum was added into the medium.

3.2.3 Callus Induction

Method applied for the production of callus basically similar to the regeneration of microshoots and root with slightly modification (explant from sterile plantlets). Leaf explants were cut into segments (1 cm x 1 cm), while stems and nodes were cut into 1 cm in length. All the explants were cultured on MS media fortified with different types, concentrations and combinations of plant growth regulators. MS media without plant growth regulators was used as control. The media tested in this study were as listed below;

1. MS basal
2. MS + 0.5 mg/l 2,4-D
3. MS + 1.0 mg/l 2,4-D
4. MS + 2.0 mg/l 2,4-D
5. MS + 0.5 mg/l NAA
6. MS + 1.0 mg/l NAA
7. MS + 2.0 mg/l NAA
8. MS + 1.0 mg/l TDZ
9. MS + 2.0 mg/l TDZ
10. MS + 2.0 mg/l 2,4-D + 1.0 mg/l BAP
11. MS + 2.0 mg/l NAA + 1.0 mg/l BAP
12. MS + 2.0 mg/l 2,4-D + charcoal
13. MS + 2.0 mg/l 2,4-D + 1.0 mg/l BAP + charcoal

14. MS + 2.0 mg/l NAA + charcoal
15. MS + 2.0 mg/l NAA + 1.0 mg/l BAP + charcoal
16. MS + 1.0 mg/l TDZ+ charcoal
17. MS + 2.0 mg/l TDZ+ charcoal
18. MS + 2.0 mg/l 2,4-D + 0.1 g/l myo-inositol
19. MS + 2.0 mg/l 2,4-D + 0.5 g/l myo-inositol
20. MS + 2.0 mg/l NAA + 0.1 g/l myo-inositol
21. MS + 2.0 mg/l NAA + 0.5 g/l myo-inositol

All cultures were kept in the culture room with photoperiod of 16 hours light and 8 hours dark.

3.3 Somatic Embryogenesis and Indirect Regeneration of *Dioscorea alata* L.

3.3.1 Establishment Phase for Callus Formation

Node explants from five-month-old intact plants of *Dioscorea alata* L. were used in this experiment. Intact plants were derived from tubers grown at garden in Institute of Biological Sciences, Faculty of Science, Universiti Malaya. Node explants were sterilized using the same steps for explant sterilization as discussed in section 3.1.1.2. After removed damages areas, each explant was cut into 1 cm x 1 cm and cultured on MS media supplemented with different concentrations and combinations of plant growth regulators.

3.3.2 Embryogenic Callus Initiation

Node explants were cultured on embryogenic callus induction media. Media was prepared using the same as for media preparation discussed in section 3.1.2. The list of media used for embryogenic callus induction are as below;

1. MS media (control)
2. MS + 0.5 mg/l NAA

3. MS + 1.0 mg/l NAA
4. MS + 1.5 mg/l NAA
5. MS + 2.0 mg/l NAA
6. MS + 2.5 mg/l NAA
7. MS + 3.0 mg/l NAA
8. MS + 0.5 mg/l 2-4,D
9. MS + 1.0 mg/l 2-4,D
10. MS + 1.5 mg/l 2-4,D
11. MS + 2.0 mg/l 2-4,D
12. MS + 2.5 mg/l 2-4,D
13. MS + 3.0 mg/l 2-4,D

The cultures were incubated in the culture room at 25 ± 1 ºC with 16 hours light and 8 hours dark. After 2 months, white purplish friable callus was observed. Callus that was obtained were analyzed whether it is embryogenic or non embryogenic using double staining technique. These callus were then transferred to other media to induce somatic embryogenesis.

3.3.3 Identification of Embryogenic Callus

Callus can be divided into two types, embryogenic and non-embryogenic callus. Embryogenic callus has the ability to regenerate into new plantlets. Embryogenic and non embryogenic callus can be identified by using 'double staining' technique following the method established by Gupta and Durzan in 1987. Initially, 2% acetocarmine and 0.5% Evan's Blue solution were prepared. Then, small pieces of callus (3-5 mm) were placed on clean glass slides. A few drops of acetocarmine was added until all callus were submerged. The callus was gently divided with forceps into very small pieces in the acetocarmine solution. The specimens were flamed or heated gently for 2 minutes without

boiling it. The callus was washed for 2 to 3 times with distilled water to remove all liquid of acetocarmine. Two drops of 0.5% Evan's Blue was added to Acetocarmine stained cells. After 30 seconds, the slides were washed 2-3 times with water and then all water was removed. One to two drops of glycerol was added to stained cells to prevent drying. The slides were then observed under light microscope with different magnifications (10X, 40X and 100X) and the embryogenic and non embryogenic callus were identified.

3.3.4 Preparation of Somatic Embryogenesis Induction Medium

Solid and liquid culture media were used for somatic embryo induction media. Solid media was prepared using the same steps as discussed in section 3.1.2 by diluting MS powder with 30 g/l sucrose and 8 g/l agar in distilled water. Liquid culture media was prepared using the same method but without the addition of gelling agent. Various types and concentrations of plant plant growth regulators such as 2,4-D and NAA were added into the culture medium to study the effects on induction and formation of somatic embryos. Solid and liquid media were supplemented with the same concentrations and combinations of plant growth regulators. Below is the list of media that was used in this study.

1. MS media (control)
2. MS + 0.5 mg/l BAP
3. MS + 1.0 mg/l BAP
4. MS + 1.5 mg/l BAP
5. MS + 2.0 mg/l BAP
6. MS + 0.5 mg/l NAA
7. MS + 1.0 mg/l NAA
8. MS + 1.5 mg/l NAA
9. MS + 2.0 mg/l NAA

10. MS + 2.5 mg/l NAA
11. MS + 3.0 mg/l NAA
12. MS + 0.5 mg/l 2-4,D
13. MS + 1.0 mg/l 2-4,D
14. MS + 1.5 mg/l 2-4,D
15. MS + 2.0 mg/l 2-4,D
16. MS + 2.5 mg/l 2-4,D
17. MS + 3.0 mg/l 2-4,D
18. MS + 2.0 mg/l 2-4,D + 0.5 mg/l BAP
19. MS + 2.0 mg/l 2-4,D + 1.0 mg/l BAP
20. MS + 2.0 mg/l 2-4,D + 1.5 mg/l BAP
21. MS + 2.0 mg/l 2-4,D + 2.0 mg/l BAP

3.3.5 Induction of Somatic Embryos

Embryogenic callus was subcultured onto solid media and liquid media for somatic embryo formation. Small pieces (0.5 cm) of embryogenic callus was transferred to solid media or liquid somatic embryogenesis induction media. Cultures in solid media were maintained in the culture room under photoperiod of 16 hours light and 8 hours dark. Cultures in liquid media were maintained on a rotary shaker at 100 rpm in the same culture room. Different stages of somatic embryogenesis were observed after 3 months.

3.3.6 Somatic Embryos Maturation and Germination

For embryo maturation and germination, somatic embryos were transferred onto somatic embryo germination media containing various concentrations of BAP and NAA. For each treatment, 2 cm x 2 cm callus with somatic embryos were cultured onto MS solid medium. Percentage of embryos germination and number of shoots and roots

formation were observed after one month of culture. Below is the list of media used in this experiment.

1. MS Basal media
2. MS + 0.5 mg/l BAP
3. MS + 1.0 mg/l BAP
4. MS + 1.5 mg/l BAP
5. MS + 2.0 mg/l BAP
6. MS + 0.5 mg/l NAA
7. MS + 1.0 mg/l NAA
8. MS + 1.5 mg/l NAA
9. MS + 2.0 mg/l NAA
10. MS + 1.0 mg/l BAP + 0.5 mg/l NAA
11. MS + 1.0 mg/l BAP + 1.0 mg/l NAA
12. MS + 1.0 mg/l BAP + 1.5 mg/l NAA
13. MS + 1.0 mg/l BAP + 2.0 mg/l NAA

3.4 Synthetic Seed Studies in *Dioscorea alata* L.

3.4.1 Preparation of Synthetic Seed Propagules

The plant materials for this experiment was obtained from *in vitro* cultures of *Dioscorea alata* derived from intact plants. Shoot tips, stems and nodes from 5-month-old complete plantlets were used as propagules for synthetic seed formation.

3.4.2 Preparation of Encapsulation Matrix

Seeds are normally produced by growing plants as a direct consequence of the sexual process. The normal seeds consist of mature embryos in a resting stage as well as various nutritive tissues (endosperm) and seed coats necessary for food storage, transport and

protection. Synthetic seeds mimic the natural seeds. The seeds consist of tissues derived from vegetative parts and artificial endosperms. Encapsulation matrix acted as the artificial endosperm. The condition of an artificial endosperm could provide nutrients and growth regulators that were essential for plantlet development from encapsulated part (Nieves et al., 1998). Usually propagules from cultures encapsulated in a nutrient gel containing essential organic or inorganic salt, carbon source, plant growth regulators, vitamin and coating agent. The pH of encapsulation matrix was adjusted to 5.8.

3.4.2.1 Preparation of MS Basal Medium (Essential organic/ inorganic salt)

MS basal medium without calcium chloride (Ca-Free MS) was prepared using MS stock solution which contain:

i) Macronutrient: Magnesium sulphate, potassium phosphate, potassium nitrate, ammonium nitrate.

ii) Micronutrient: Boric acid, Manganese sulphate, Zink sulphate, Sodium Molybdate, Copper sulphate, Cobalt (II) Chloride Hexahydrate, Potassium Iodide (KI).

iii) Iron: Ferrous sulfate heptahydrate, Disodium ethylenediaminetetraacetate dihydrate.

iv) Vitamin: Thiamine HCL, Pyridoxine HCL, and Nicotinic acid.

v) Myo-inositol.

vi) Sucrose.

3.4.2.2 Preparation of Sodium alginate solution (Coating agent)

Sodium alginate ($NaC_6H_7O_6$) consists of alginate acid was used as the coating agent in encapsulation matrix of synthetic seed. Sodium alginate solution was prepared using a heating method used by Fabre and Dereudde (1990). To prepare 1.0% sodium alginate

solution, 1.0 g of sodium alginate powder was added into 100 ml MS basal medium without calcium chloride dehydrate (Ca-Free MS), then 3g sucrose was added, pH was adjusted to 5.8. Finally autoclaved for 21 minutes at 121ºC.

3.4.3 Preparation of Calcium Chloride (Hardening the encapsulation matrix)

Calcium chloride dehydrate ($CaCl_2.2H_2O$) was used for hardening the encapsulated seed. Hardening treatment was imposed in order to block precocious germination and to increase encapsulated explants survival after desiccation. To prepare 50 mM Calcium chloride dehydrate solution, 1.8378 g of Calcium chloride dehydrate powder was diluted in 250 ml distilled water and autoclaved for 21 minutes at 121ºC.

3.4.4 Encapsulation Technique of Synthetic Seed

Encapsulation technique was used to prepare synthetic seed. Shoot tips, node and stem explants were isolated from cultures and blot dried on filter paper. After that, explants were placed into Sodium alginate solution. To form one bead, micropipette size 5mm was used to draw one explant with sodium alginate solution and dropped into Calcium chloride solution. After 40 minutes, decanting the Calcium chloride solution from the beads and the beads were washed 3 times with sterile distilled water.

3.4.5 Preparation of the Regeneration Medium

MS solid and liquid media were used to germinate the synthetic seed produced. MS solid media was prepared using the same steps as media preparation discussed in section 3.1.2. MS liquid media was prepared using the same method without adding gelrite gellan gum. All cultures were maintained in culture room at 25±1º C under16 hours light and 8 hours dark. The survival rate was recorded. Thirty replicates were used in each treatment.

3.4.6 Preparation of Sodium alginate and Calcium Chloride Dehydrate Solution

Four different concentrations of sodium alginate solution and four different concentrations of Calcium Chloride Dehydrate Solution were prepared. Four different concentrations of sodium alginate were prepared which are 2.0%, 3.0%, 4.0% and 5%. Four concentration of Calcium chloride were prepared; 25mM, 50 mM, 75 Mm and 100 mM.

3.4.7 Effects of Plant growth regulators in Sodium alginate Solution

Sodium alginate solution at concentration of 3% was prepared with addition of plant growth regulators. Two types of solution were prepared i.e. 3% sodium alginate supplemented with 1.0 mg/l BAP and 3% sodium alginate supplemented with 1.0 mg/l BAP and 2.0 mg/l 2,4-D.

3.4.8 Effects of Low Temperature Storage Period on Germination of Synthetic seeds

Sterile synthetic seeds (encapsulated shoot tips) in 3% sodium alginate and 75 mM calcium chloride dehydrate) covered with aluminum foil (dark condition) were stored in a laboratory refrigerator at 4±1º C. At 7 days intervals up to 30 days, the synthetic seeds were inoculated and cultured on MS solid and in liquid media maintained in culture room at 25±1º C under16 hours light and 8 hours dark. The survival rates were recorded. Thirty replicates were used in this treatment.

3.5 Acclimatization of *In vitro* Plantlets of *Dioscorea alata* L.

3.5.1 Soil Analysis

Growth substrates play as a crucial factor in acclimatization process. In order to establish the optimum growth of micropropagated plantlets transferred, substrates were analysed to determine the Exchangeable Bases (K, Ca and Mg), Cation Exchange

Capacity (CEC), Nitrogen (N), Phosphorous (P), Potassium (K), Total Phosphorous, Available Phosphorous and moisture content of the substrates. Experiments were conducted at

Macronutrients (N, P, K, Ca and Mg) were analyzed using Atomic Absorption Sectrophotometer (AAS). The exchangeable bases and CEC was analyzed using leaching method. First, 10 g of substrate was weighed out and packed into leaching column. After that, 100 ml of 1M Ammonium Acetate reagent was added into 350ml Erlenmeyer flask fitted with the outlet tube and invert over the soil column. After 5 hours, leachates were collected and the solution with 1M Ammonium Acetate for the determination of K, Mg and Ca. The remaining sample was washed with spirit metal for determination of Cation Exchange Capacity (CEC). The sample was titrated with 0.075N HCl until the green colour turn to light purple. The volume of HCl used was recorded. The basic calculation for CEC value ($cmol_c$/ kg) is:

CEC value: (V_{HCL} of sample – V_{HCL} of black) X 0.021 X (1000/14)

Total Nitrogen content was analyzed using Kjeldahl method by using AAS. One tablet Kjeldahl catalyst and half spatula of sodium thiosulphate was added with 1.0 g soil into digestive tube of AAS. 25 ml boric acid and 3 drops of methyl alcohol was added into the mixture. Then, the solution was titrated with 0.075N HCl using TitraMate 10 METTLER TOLEDO. The volume of HCl used was recorded. The basic calculation for Total N are:

$$\% \text{ N} = \frac{14.01\ X\ (ml\ titrant - ml\ blank) - (N\ of\ titrant) X\ 100}{sample\ weight\ (g) X\ 1000}$$

The total carbon was determined using Dry Combustion Method with Leco Trumac-Determinations Version 1.1 x CNX analyzer. Total P was analysed using AAS. 1.0 g soil

was added into 3 ml of mixture of sulphuric acid and perchloric acid with a ratio of 50: 50. Reagent C was added into the solution. After that the sample was analysed using UV Spectrophotometer. Available phosphorous was determined by using Bray II Method (Anon, 1980). Two g of air dried soil were weighed out and put into plastic vial. After that, 20 ml of Available phosphorous extraction solution was added into the sample. The sample was stirred using orbital shaker for 30 min and the final product was analyzed by using UV Spectrometer for determination of Available P in the soil.

3.5.2 Plant Materials

Complete micropropagated plantlets obtained from the *in vitro* regeneration was used in this experiment. After 4 months, the new plantlets obtained from *in vitro* cultures were ready to be transferred to the natural environment as they developed sufficient shoots and roots.

3.5.3 Growing Substrates and Transferring Plantlets to *ex vitro* Environment

Well rooted four-month-old micropropagated (*in vitro*) plantlets were carefully removed from culture tubes and roots were washed thoroughly under running tap water to remove the adhering gel and all the medium traces. They were then transplanted to plastic pots (80 x 60 mm) containing acclimatization media with different scheme of growth medium; namely Black soil, Red soil, Mixture of Black soil and Red soil at ratio 2 to 1. The potted plantlets were then covered with transparent plastics with small holes to reduce water lost. The potted plantlets were first kept in the culture room at 25 ± 1 °C under 16 hours light and 8 hours dark for 4 weeks before exposing to the natural environment. The survival rates were recorded after 4 and 8 weeks of transfer. After 4 weeks in culture room, potted plantlets were transferred to green house. Shading was used to reduce irradiance during the first day of acclimatization. After 30 days in the

greenhouse, the plastic cover was slowly lifted up and the shading of the covered reduced. At day 60 the plastic covered was removed completely. Based on all experiments done, the best acclimatization technique which gave the highest survival rate was identified.

3.5.4 Measurement of Agronomic Parameters

Several agronomic parameters such as plant height, number of leaves, and number of nodes per plant were measured after 4 and 8 weeks micropropagated plantlets being acclimatized.

3.6 Anthocyanin Content and Accumulation in *Dioscorea alata* L.

3.6.1 Preparation of Samples

Fresh tubers and leaves obtained from intact plant (*in vivo* grown) of *Dioscorea alata* were used as *in vivo* samples and callus and plantlets from tissue culture technique were used as *in vitro* samples.

***In vivo* samples**

Tubers (cleaned, peeled and sliced into 5 x 5 x 1 cm^3 cube) and leaves of *Dioscorea alata* were freeze dried for one week. After that, samples were pulverized to powder form by using electric grinder. Required quantity of powder were weighed out and treated with methanol until the powder is fully immersed. The mixture was shaken for 1 hour and centrifuged at 4000 rpm for 15 min.

***In vitro* samples**

Purple coloured callus and plantlets of *Dioscorea alata* were induced using different combination of plant growth regulators. Node explants were cultured on MS (Murashige and Skoog, 1962) media containing 30 g/l sucrose and 3.5 g/l gelrite gellan gum. All cultures were kept in the culture room with the photoperiod 16 hours light and 8 hours dark. Callus and plantlets obtained from tissue culture of *Dioscorea alata* were freeze

dried for one week. After that, samples were pulverized to powder form by using electric grinder. Required quantity of powder were weighed out and treated with methanol until the powder is fully immersed. The mixture was shaken for 1 hour and centrifuged at 4000 rpm for 15 min.

3.6.2 Extraction of Pigments

Samples (10.0 g) were dissolved in 100 ml methanol solution to extract the colour (anthocyanin). The mixtures were then centrifuged at 5000 rpm for 10 min. Subsequently, the supernatant was collected and filtered through Whatman No. 1 filter paper to remove any residues. Finally, the extracts were covered with aluminum foil and stored under dark condition at 4±1 ºC to avoid colour degradation and hydrolysis of the acyl groups in the anthocyanin structure.

3.6.3 Total anthocyanin Content Measurement

The total anthocyanin content were estimated according to the spectrophotometric pH differential method (Gabriela et al., 2010; Lee et al., 2005). Sample were diluted separately with 0.025 M potassium chloride buffer (pH 1) and 0.4 M sodium acetate buffer (pH 4.5). Absorbance of the mixture was measured at 530nm ($\lambda_{vis-max}$) and 700nm using a UV-Vis spectrophotometer. The total anthocyanin content was calculated as cyaniding-3-glucoside equivalent following the equation:

$$\text{Anthocayanin content } \left(\frac{mg}{100g}\right) = \frac{[A \times MW \times DF \times V \times 100]}{\varepsilon \times l \times m}$$

Where,

A is the absorbance, calculated as A= [($A_{511} - A_{700}$) pH 1.0 – (A_{511}- A_{700}) pH 4.5].MW is the molecular weight of 449.2 g/mol of cyaniding-3-glucoside.

DF is the dilution factor

V is solvent value (ml) that was brought sample stock solution

ε is the molar absorptivity (26900)

l is the cell path length (1 cm).

m is the sample weight (g).

3.6.4 Effect of Light/ Dark Cycle on the Anthocyanin Production (Photoperiod)

Plant organs (leaf, stem, and node) sectioned into small pieces were utilized as the explants to examine the effects of light on coloured callus production. The explants were cultured on the optimum medium (MS medium supplemented with 2.0 mg/l 2,4-D added with 0.5 mg/l myo-inositol) and maintained in the culture room under different light treatments (photoperiod) which are:

i) 0 hour light and 24 hours dark (dark)

ii) 12 hours light and 12 hours dark

iii) 16 hours light and 8 hours dark

iv) 24 hours light 0 hour dark

3.6.5 Estimation of Total Phenolic Content

The total phenolic content were estimated using Folin-Ciocalteu method. The procedure was performed according to Lachman et al. (2000). Samples (100 µL) were mixed with 2 ml of Na_2CO_3. After 2 min, 100 µL of Folin-Ciocalteu reagent was added to the mixture. The resulting mixture was incubated for 30 min at room temperature then absorbance was read spectrophotometrically at 743 nm against a blank. Total phenolic was expressed as gram of gallic acid equivalent per 100 gram of dry weight (g $100g^{-1}$DW) of the plant samples.

3.6.6 Estimation of Flavonoid Content

An aliquot of 0.5ml of sample (1mg/mL) was mixed with 0.1ml of 10% aluminium chloride and 0.1% of potassium acetate (1M). 4.3ml of 80 % methanol was added into the mixture to make 5ml volume. Mixture was vortexed. Absorbance was measured

spectrophotometrically at 415nm. The value of optical density was used to calculate the total flavonoid content in the sample.

3.7 Antimicrobial Potential in *Dioscorea alata* L. *In vivo* and *In vitro Samples*

3.7.1 Preparation of Plant Extract

For ethanolic extracts, about 5.0 g fresh *in vivo* grown tuber and *in vitro* leaves and callus samples of *Dioscorea alata* were weighed out. The samples were then crashed in a mortar. A 20 ml volume of aqueous ethanol 95% was added and the samples were homogenized and centrifuged at 5000 rpm for 15 min. Supernatant was collected and used for antimicrobial test. For water extract, same step was repeated but ethanol was replaced with distilled water.

3.7.2 Preparation of the Microbe

Eight different microbial species were used to screen the possible antimicrobial activity of *Dioscorea alata* from *in vivo* and *in vitro* samples, alcoholic extract. Four bacteria, *Staphylococcus aureus*, *Staphylococcus epidermis*, *Escherichia coli* and *Salmonella sp.* were tested for antibacterial. Four different fungi which are *Penicillium sp.*, *Aspergilus niger*, *Fusarium sp.* and *Mucor sp*. were tested for antifungal.

3.7.3 Preparation of the Media

3.7.3.1 Preparation of Media for Bacterial culture

Nutrient Broth (NB) was used to inoculate the bacteria and Nutrient agar (NA) media was used to culture bacteria. Nutrient Broth 'E' from LAB, United Kingdom was prepared by dispersed 13 g of NB in 1 litre distilled water. NA was prepared by diluting NA powder in distilled water. 23 g Difco TM NA powder from Difco Laboratories, France was weighed out and dispersed in 1 litre of distilled water. The powder was mix thoroughly,

heat with frequent agitation and boil for 1 minute to completely dissolve the powder. After that, the media was autoclaved at a pressure of 104 kPa (15 Psi^2) and temperature of 121ºC for 20 minutes. After the media has been autoclaved and cooled (50ºC), the media were dispensed into 14 cm sterile petri dishes.

3.7.3.2 Preparation of Media for Fungal Culture

Potato Dextrose Agar (PDA) media was used to culture fungi. PDA was prepared by diluting PDA powder in distilled water. To prepare the media, 39 g of PDA from Oxoid, England was weighed out and disperse in 1 litre distilled water. The media solution was stirred until all the PDA powder was dissolved. All the media was autoclaved at a pressure of 104 kPa (15 Psi^2) and temperature of 121ºC for 20 minutes. After the media has been autoclaved and cooled (50ºC), the media were dispensed into 14 cm sterile petri dishes.

3.7.4 Microbe Cultures

3.7.4.1 Bacterial Cultures

The antibacterial test was made using 24 hours old broth cultures (overnight cultures). To prepare the cultures, one loop of each bacteria was placed into 100 ml NB media, respectively and inoculated for 24 hours. After 24 hours, a series of dilution were done. 1 ml of the bacteria broth was pipet into a centrifuge tube contained 9 ml saline (prepared by diluting sodium alginate 13g in 1 litre distilled water). The solution was shaken for 1 min. After that, 1 ml of this solution, was pipetted into another centrifuge tube contained 9 ml saline. This step was repeated 7 times. Then, 10 µl of the last solution was pipetted onto NA media and spread using hockey stick.

3.7.4.2 Fungal Cultures

To prepare the cultures, one loop of filamentous fungi was inoculated in 250 ml Erlenmeyer flask with 100 ml malt extract broth media (15 g malt extract and 5 g peptone in 1 litre distilled water) and incubated for 24 hours. After 24 hours, a series of dilution were done. 1 ml of the fungi broth was pipetted into a centrifuge tube containing 9 ml saline (prepared by diluting sodium alginate 13g in 1 litre distilled water). The solution was shaken for 1 minute. After that, 1 ml of this solution, was pipeted into another centrifuge tube contained 9 ml saline. This step was repeated 7 times. Then, 10 µl of the last solution was pipetted onto PDA media and spread using hockey stick.

3.7.5 Antimicrobial Test (Paper Disk Diffusion Method)

Sterile paper disks (6.0 mm in diameter) were impregnated with 20 µl of *in vivo* and *in vitro* alcoholic extract. The disks were allowed to dry for 5 min. After that, three disks were spaced on the microbe media surface of each Petri dish. The diameter of the inhibition zone around the disks was measured after 48 hours incubation at 30 ºC.

3. 8 Statistical Analysis

Three replications with 30 explants in each replication were maintained for each treatment. Data obtained was analyzed statistically using one way ANOVA and Duncan's Multiple Range Test (DMRT). The statistical analysis based on mean values per treatment was made using the technique of analysis of variance. The comparative LSD *multiple range test* (p=0.05) was used to determine the differences between treatments.

CHAPTER 4: RESULTS

4.1 Regeneration of *Dioscorea alata* L. *in vitro*

4.1.1 Effect of light on seedlings derived from tubers germination and conversion

Light play crucial role in tuber germination and conversion. Observations were made after 2 months of tubers planting. Data presented in Table 4.1 showed, 100.00± 0.00 percent of tubers were successfully germinated in all treatment. All 100% germinated tubers exposed under direct sunlight managed to grow with 151.00± 0.37 cm in height and developed healthy leaves. Tubers (100%) grown under light provided by fluorescent tubes managed to growth well with 50.40± 0.27 cm in height and developed into leaves. Ovule, narrow heart shape and green with slightly purple colour leaves were observed on plant grown in green house, 24.00 ± 0.30. Ovule, narrow heart shape and purple colour leaves were observed on plant grown under fluorescent tube light, 6.50 ± 0.27 (Figure 4.1). However, in dark condition only 80.00±0.13 % of tubers managed to sprout and grow with 17.00 ± 0.26 cm in height without leaf formation. Some of the germinated bud became browning and black. After 6 weeks, necrosis occurred to the bud. In term of plant growth and height, direct sunlight is the best condition for growing *Dioscorea alata*.

Although plants grown under direct sunlight showed positive growth to provide plenty of explant sources (more leaves, longer stem and many node) but plant grown in the laboratory under light supplied by fluorescent tubes was chosen as the explant sources for tissue culture work because of the sterile conditions. Plant grown in green house were exposed to dust and some contamination which make it difficult to sterilize the explants effectively during sterilization technique prior to culturing the explants in the sterile containers.

Table 4.1: Effects of light on tubers conversion of *Dioscorea alata* L.

Plant characteristics	Observations		
	Greenhouse (Direct Sunlight)	Laboratory (with an irradiance of 50 µmol $m^{-2}s^{-1}$ provided by Philips Lifemax TL-D 18W cool daylight fluorescent tubes)	No light (covered with black plastic)
Conversion rate (%)	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]
Growth rate (%)	100.00±0.00[a]	100.00±0.00[a]	80.00±0.13[a]
Height (cm)	151.00±0.37[a]	50.40±0.27[b]	17.00±0.26[c]
Leaves			
No of leaves:	24.00±0.30[a]	6.50±0.17[b]	0.00±0.00[c]
Shape:	Ovule, narrow heart shape leaves.	Ovule, narrow heart shape leaves.	-
Colour:	Green with slightly purple.	Purple	-
Stem			
Length (cm):	151.00±0.37[a]	50.40±0.27[b]	17.00±0.26[c]
Shape:	Cylindrical, twining herbaceous vine with internodes square in cross section.	Cylindrical, twining herbaceous vine with internodes square in cross section	Cylindrical, twining herbaceous vine with internodes square in cross section
Colour:	Purple tinged with more green colour.	Purple tinged with slightly green colour	Purple.

Mean values with different letters within a column are significantly different at p=0.05, by one way ANOVA and Duncan's Multiple Range Test (DMRT).

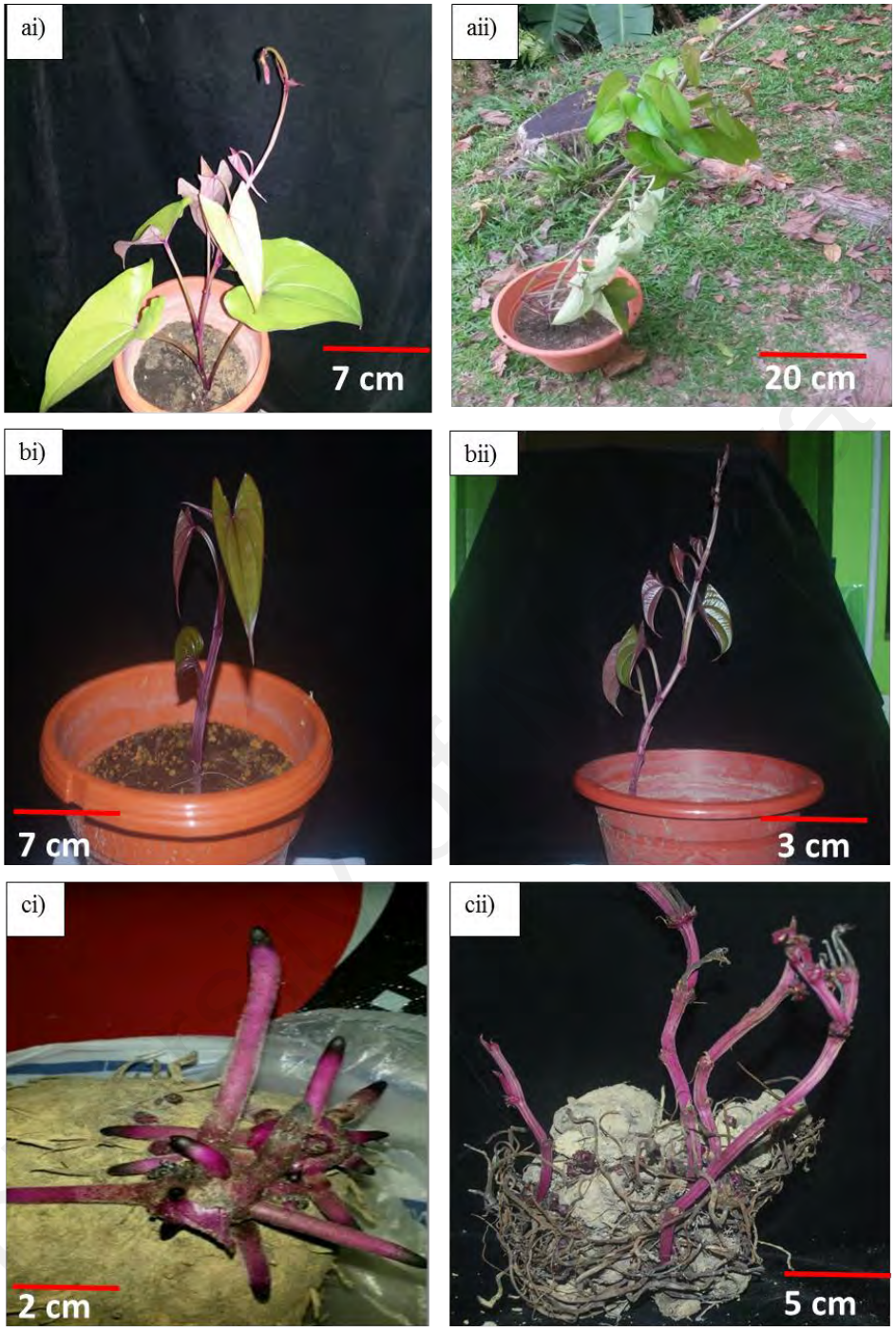


Figure 4.1: Tubers conversion in black soil maintained at different light conditions. ai) Two-week-old plantlet germinated under direct sunlight, aii) Three-month-old plantlet germinated under direct sunlight, bi) Two-week-old plantlet germinated under an irradiance of 50 µmol $m^{-2}s^{-1}$ provided by Philips Lifemax TL-D 18W cool daylight fluorescent tubes, bii) Three-month-old plantlet germinated under an irradiance of 50 µmol $m^{-2}s^{-1}$ provided by Philips Lifemax TL-D 18W cool daylight fluorescent tubes ci) Two-week-old plantlet germinated without light and cii) Three-months-old plantlet germinated without light .

4.1.2 Identification of suitable plant growth regulators and types of explants for microshoots formation

Microshoots formation from *in vitro* regeneration of *Dioscorea alata* were observed after 4 months in all the tested media. Table 4.2 shows the response of leaf, stem and node explants of *Dioscorea alata* when cultured on MS media supplemented with BAP and NAA at various combinations and concentrations. Leaf and stem explants showed negative response in terms of shoot formation in all treatments. No shoot formation was observed from leaf and stem explants. Only node explants successfully regenerated into shoots. Node explants have axillary buds or lateral buds within. Each bud has the potential to form shoots. Enlargement and subsequent break of axillary buds was the initial response of node explants. The axillary bud on the node showed visible growth after 7 days in culture. Based on the results, node explants cultured in medium without plant growth regulators or MS basal, successfully produced 4.63±0.24 mean shoots after 8 weeks in culture (Figure 4.2). The highest shoot regeneration (23.07±0.44) was observed from node explants cultured on MS medium supplemented with 1.0 mg/l BAP.

Lower percentage of shoots was obtained from node explants cultured in MS media supplemented with NAA alone. Only 0.50±0.12 average number of shoot was observed in MS medium supplemented with 3.0 mg/l NAA. However, in MS media supplemented with NAA in combination with BAP significant higher number of shoots was observed. Of all the combination tested, MS medium supplemented with 1.0 mg/l BAP and 0.5 mg/l NAA showed optimal response with mean number of shoot formation 21.40±0.33.

Table 4.2: Mean number of microshoots formation from leaf, stem and node explant cultured on MS media supplemented with different concentrations and combinations of plant growth regulators maintained at 25±1^{0}C with 16 h light and 8 h dark. Thirty replicates were used in each treatment.

MS + Plant Growth Regulators (mg/l)	Mean number of micro shoots per explant		
	Leaf	Stem	Node
MS Basal	NR	NR	4.63±0.24^{lm}
MS+0.5 NAA	NR	NR	4.33±0.20^{lmn}
MS+1.0 NAA	NR	NR	2.27±0.17^{p}
MS+1.5 NAA	NR	NR	1.07±0.24^{qr}
MS+2.0 NAA	NR	NR	1.90±0.20^{pq}
MS+3.0 NAA	NR	NR	0.50±0.12^{r}
MS+0.5 BAP	NR	NR	17.27±0.36^{d}
MS+1.0 BAP	NR	NR	22. 00±0.43^{b}
MS+1.5 BAP	NR	NR	21.40±0.33^{b}
MS+2.0 BAP	NR	NR	16.83±0.42^{d}
MS+3.0 BAP	NR	NR	7.97±0.30^{i}
MS+0.5 NAA+0.5 BAP	NR	NR	13.33±0.31^{f}
MS+0.5 NAA+1.0 BAP	NR	NR	23.07±0.44^{a}
MS+0.5 NAA+1.5 BAP	NR	NR	18.30±0.34^{c}
MS+0.5 NAA+2.0 BAP	NR	NR	15.10±0.31^{e}
MS+0.5 NAA+3.0 BAP	NR	NR	11.33±0.24^{g}
MS+1.0 NAA+0.5BAP	NR	NR	2.03±0.21^{p}
MS+1.0 NAA+1.0 BAP	NR	NR	16.63±0.40^{d}
MS+1.0 NAA+1.5 BAP	NR	NR	12.53±0.27^{f}
MS+1.0 NAA+2.0 BAP	NR	NR	14.83±0.40^{e}
MS+1.0 NAA+3.0 BAP	NR	NR	18.57±0.35^{c}
MS+1.5 NAA+0.5BAP	NR	NR	3.20±0.33^{o}
MS+1.5 NAA+1.0BAP	NR	NR	6.50±0.26^{j}
MS+1.5 NAA+1.5BAP	NR	NR	10.03±0.33^{h}
MS+1.5 NAA+2.0BAP	NR	NR	9.20±0.40^{h}
MS+1.5 NAA+3.0BAP	NR	NR	8.17±0.20^{i}
MS+2.0 NAA+0.5BAP	NR	NR	3.50±0.19^{no}
MS+2.0 NAA+1.0BAP	NR	NR	4.40±0.21^{lmn}
MS+2.0 NAA+1.5BAP	NR	NR	3.67±0.22^{mno}
MS+2.0 NAA+2.0BAP	NR	NR	4.97±0.30^{kl}
MS+2.0 NAA+3.0BAP	NR	NR	5.57±0.30^{k}
MS+3.0 NAA+0.5BAP	NR	NR	4.37±0.33^{lmn}
MS+3.0 NAA+1.0BAP	NR	NR	4.50±0.32^{lm}
MS+3.0 NAA+1.5BAP	NR	NR	3.80±0.30^{mno}
MS+3.0 NAA+2.0BAP	NR	NR	3.93±0.26^{mno}
MS+3.0 NAA+3.0BAP	NR	NR	4.63±0.26^{lm}

Mean values with different letters within a column are significantly different at p=0.05, by one way ANOVA and Duncan's Multiple Range Test (DMRT). Nr=no response.

4.1.3 Identification of suitable plant growth regulators and types of explants for root formation

The well-developed elongated shoots were detached from the shoots clump and transferred to rooting induction media. Table 4.3 shows the response and development of rooting of *Dioscorea alata* shoots when subcultured in root induction media. From the results, explants successfully produced roots in all the media tested. High number of roots, 6.63±0.61 were obtained from explants cultured in MS medium without plant growth regulators. Explant cultured in MS media supplemented with BAP at all concentrations produced low number of roots compared to control. The lowest root formation, 1.57±0.21 was observed when shoots were cultured on MS medium supplemented with 2.0 mg/l BAP. However, rooting was better in the culture media which had combination of BAP and NAA. Higher number of root formation (17.27±0.61) was observed in MS medium supplemented with 2.0 mg/l NAA and 0.5 mg/l BAP. MS medium supplemented with 2.0 mg/l NAA is the most suitable medium for root formation (Figure 4.2) which gave 17.40±0.58 number of roots formation.

Table 4.3: Mean number of root formation from explant of *Dioscorea alata* L. cultured on MS media supplemented with different concentrations and combinations of plant growth regulators maintained at 25±1^{0}C with 16 h light and 8 h dark. Thirty replicates were used in each treatment.

MS + Plant Growth Regulators (mg/l)	No of root per explant (mean ± SE)
MS Basal	6.63±0.61^{g}
MS+0.5 NAA	9.43±0.45^{g}
MS+1.0 NAA	11.77±0.37
MS+1.5 NAA	12.87±0.45^{cde}
MS+2.0 NAA	17.40±0.58^{a}
MS+0.5 BAP	3.17±0.32^{ij}
MS+1.0 BAP	3.13±0.38^{ij}
MS+1.5 BAP	2.60±0.32^{j}
MS+2.0 BAP	1.57±0.21^{j}
MS+0.5 NAA+0.5 BAP	6.47±0.47^{g}
MS+0.5 NAA+1.0 BAP	5.87±0.49^{gh}
MS+0.5 NAA+1.5 BAP	4.63±0.49hi
MS+0.5 NAA+2.0 BAP	4.33±0.46^{hi}
MS+1.0 NAA+0.5BAP	11.13±0.51^{cdef}
MS+1.0 NAA+1.0 BAP	10.30±0.51^{ef}
MS+1.0 NAA+1.5 BAP	10.27±0.56^{ef}
MS+1.0 NAA+2.0 BAP	11.03±0.71^{def}
MS+1.5 NAA+0.5BAP	12.60±0.56^{cd}
MS+1.5 NAA+1.0BAP	10.83±0.68^{def}
MS+1.5 NAA+1.5BAP	11.20±0.60^{cdef}
MS+1.5 NAA+2.0BAP	10.87±0.51^{def}
MS+2.0 NAA+0.5BAP	17.27±0.61^{a}
MS+2.0 NAA+1.0BAP	15.20±0.61^{b}
MS+2.0 NAA+1.5BAP	12.13±1.03^{cd}
MS+2.0 NAA+2.0BAP	12.20±0.91^{cd}

Mean values with different letters within a column are significantly different at p=0.05, by one way ANOVA and Duncan's Multiple Range Test (DMRT).

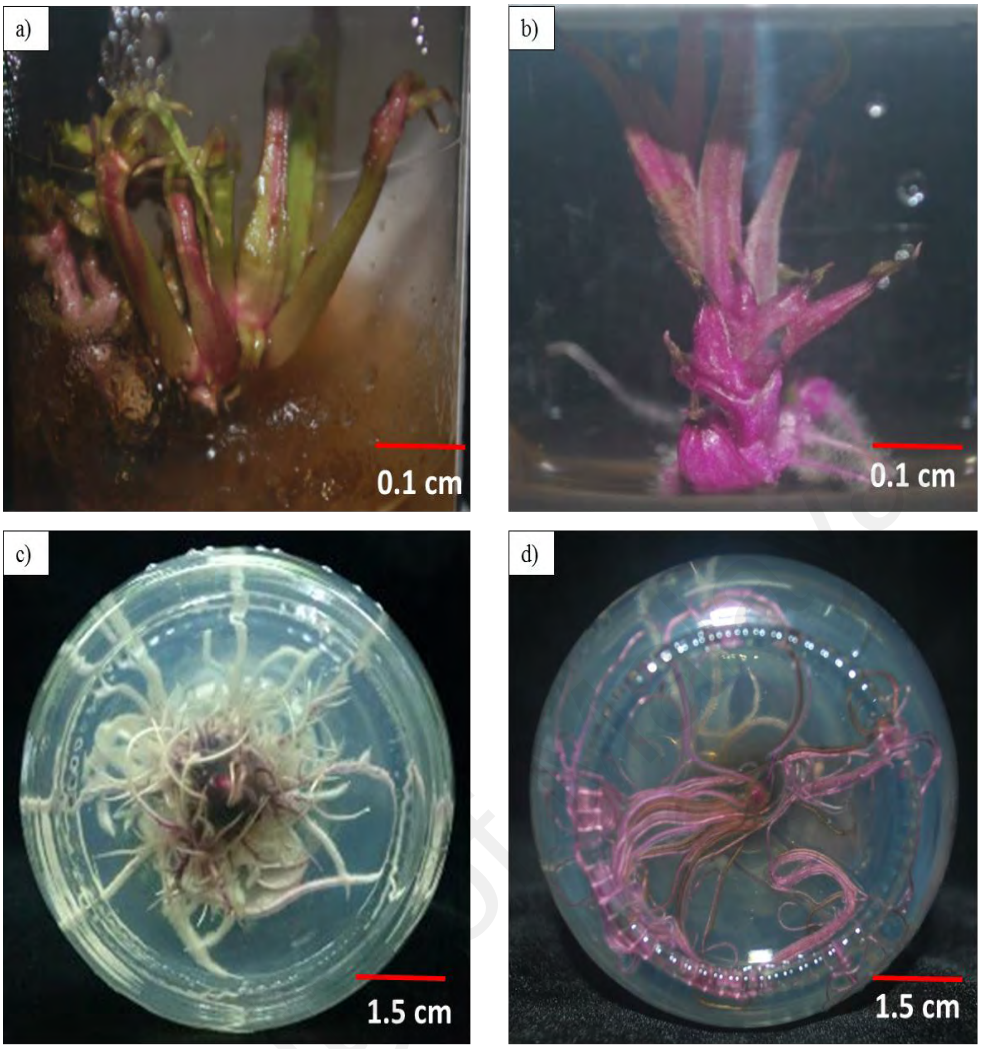


Figure 4.2: a) Microshoots formation in MS medium supplemented with 1.0 mg/l BAP, b) Microshoots formation in MS medium supplemented with 1.0 mg/l BAP and 0.5 mg/l NAA, c) Roots formation in MS medium supplemented with 2.0 mg/l NAA and d) Roots formation in MS medium supplemented with 2.0 mg/l NAA and 0.5 mg/l BAP.

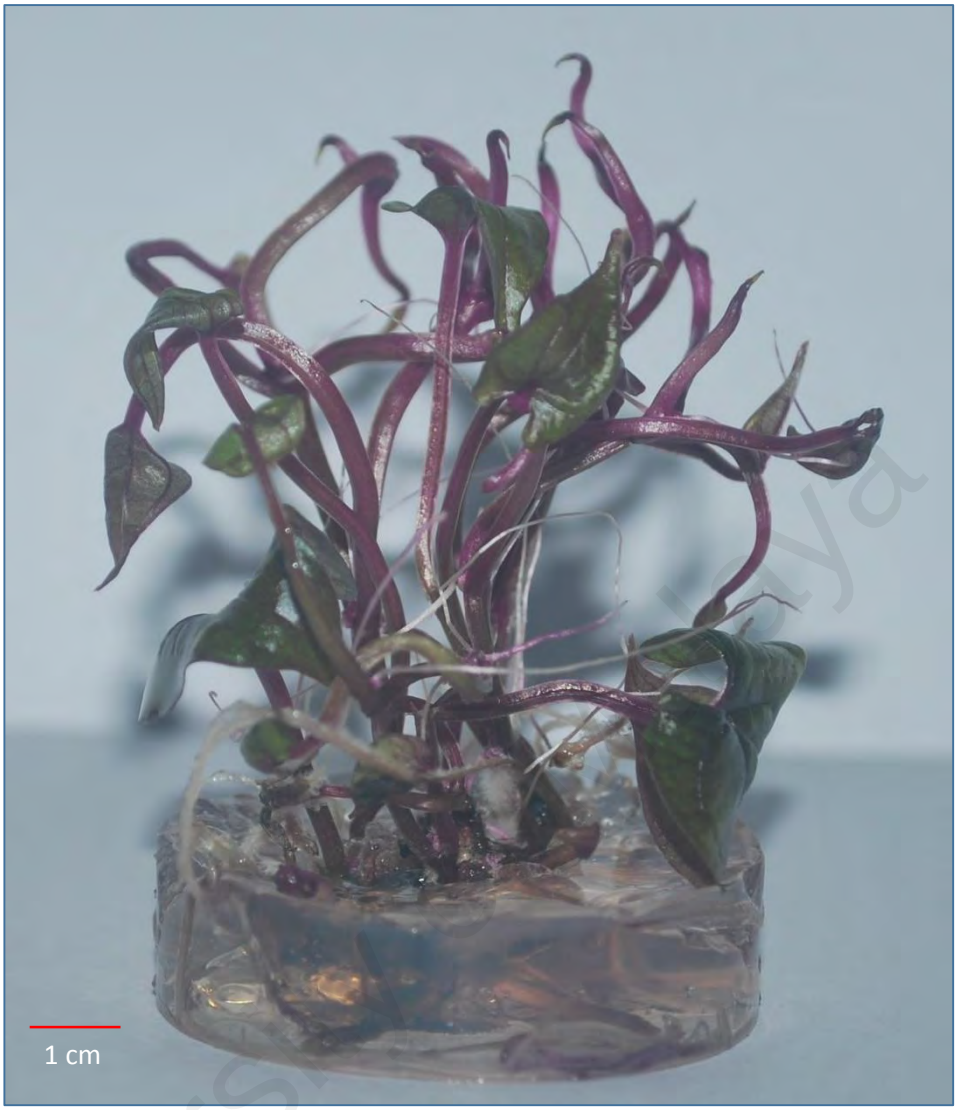


Figure 4.3: Four-months-old complete plantlet with leaf and roots obtained in MS medium supplemented with 1.0 mg/l BAP and 0.5 mg/l NAA.

4.1.4 Identification of suitable plant growth regulators for microtubers formation

Microtubers were formed on complete plantlets after 6 months in culture. In all cases where microtuberization occurred in *Dioscorea alata* cultures during the current study, microtubers were produced predominantly at basal nodal position on plantlets below culture media. Only after 8 to 10 months did microtubers occasionally developed at nodal positions above culture media. In general, microtubers were rough in surface texture and irregular in shape.

Microtubers induction (percentage of plantlets with microtubers) were influenced by plant growth regulators. Role of cytokinins were observed individually or in combinations with auxin (Table 4.4). Higher percentage of microtubers were observed on MS media supplemented with BAP compared to MS media supplemented with Kin at the same concentration. At concentration of 1.0 mg/l, 60% plantlets produced microtubers in MS media supplemented with BAP while 40% percent in MS media supplemented with Kin. The highest percentage of microtubers formation 76.67±0.08 was observed on MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA.

Data presented in Table 4.4 indicated that MS media supplemented with BAP, was the best media for microtubers formation with highest mean number of microtubers, 10.77±0.28. Additional NAA at concentration of 0.5 mg/l and 1.0 mg/l into MS medium supplemented with 1.0 mg/l BAP produced more microtubers compared to MS medium supplemented with 1.0mg/l BAP alone. MS media supplemented with Kin have less number of microtubers formation compared to control.

Microtubers development (average fresh weight and average length of microtuber) were influence by plant growth regulators. Microtubers produced in MS media

supplemented with BAP were bigger and longer compared to microtubers produced in MS media supplemented with Kin. The largest microtubers produced was observed in MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA with average length of 10.00±0.45 mm and average weight of 398±0.03 mg (Figure 4.4).

Table 4.4: Microtubers formation from plantlets of *Dioscorea alata* L. cultured on MS media supplemented with various combinations and concentrations of plant growth regulators maintained at 25±1^{0}C with 16 h light and 8 h dark. Thirty replicates were used for each treatment.

MS + Plant Growth Regulators (mg/l)	Plantlets produced microtubers (%)	No. of microtubers per plantlet	Averange length of microtubers (mm)	Averange weight of microtubers (mg)
MS Basal	40.00±0.09^{bc}	7.17 ±0.21^{e}	7.00±0.28^{de}	143±0.01^{e}
MS+1.0 BAP	60.00±0.09^{ab}	10.28±0.21^{c}	9.83±0.22^{ab}	219±0.01^{cd}
MS+1.5 BAP	43.33±0.09^{bc}	8.00±0.23^{d}	7.23±0.26^{de}	139±0.01^{e}
MS+2.0 BAP	30.00±0.09^{c}	10.77±0.28^{c}	8.11±0.26^{cd}	230±0.01^{c}
MS+1.0 Kin	40.00±0.09^{bc}	6.61±0.39^{f}	7.08±0.23^{de}	160±0.01^{de}
MS+1.5 Kin	40.00±0.09^{bc}	6.08±0.26^{f}	7.08±0.26^{de}	148±0.01^{e}
MS+2.0 Kin	23.33±0.08^{c}	3.71±0.36^{g}	6.57±0.20^{e}	193±0.02^{cde}
MS+1.0 BAP+0.5 NAA	63.33±0.09^{ab}	12.37±0.26^{b}	8.74±0.44^{bc}	326±0.01^{b}
MS+1.0 BAP+1.0 NAA	76.67±0.08^{a}	15.57±0.18^{a}	10.00±0.45^{a}	398±0.03^{a}

Mean values with different letters within a column are significantly different at p=0.05, by one way ANOVA and Duncan's Multiple Range Test (DMRT).

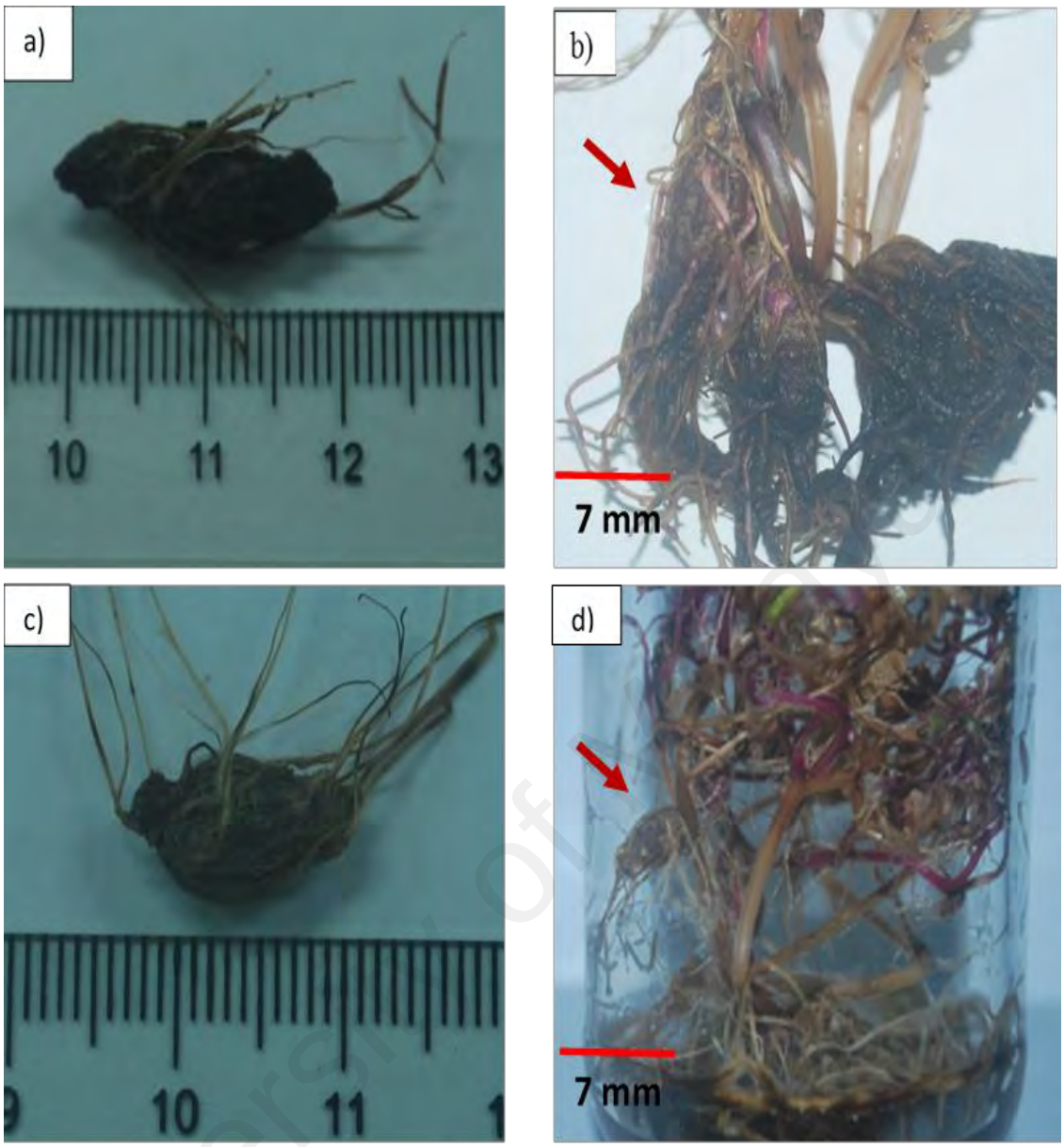


Figure 4.4: Microtubers formation on plantlets cultured on MS medium supplemented with 1.0 mg/l NAA and 1.0 mg/l BAP, a) and b) Microtubers from basal nodal position and c) and d) Microtubers from nodal position above culture media.

4.1.5 Identification of suitable plant growth regulators for microtubers conversion

Table 4.5 shows the response of microtubers as explants when cultured on MS media supplemented with BAP and NAA at various concentrations. Observations were made on the percentage of microtubers germinated, number of micro shoots per plantlet and number of roots per plantlet. Based on the results obtained in Table 4.5, more than 50 percent microtubers managed to germinate in all the media tested. 66.67±0.09 percent microtubers germinated in MS basal media. The highest percentage of microtubers germinated, 80.00±0.07 % was observed in MS medium supplemented with 2.0 mg/l NAA; MS medium supplemented with 1.0 mg/l BAP and 0.5 mg/l NAA; MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA and MS medium supplemented with 1.0 mg/l BAP and 2.0 mg/l NAA.

Microtubers explants were successfully germinated and produced shoots (Figure 4.5) in all the 16 media tested. From the Table 4.5, 4.70 ±0.18 number of shoots were observed from microtubers cultured on MS basal medium (control). Microtubers cultured on MS media fortified with BAP produced higher number of shoots and microtubers cultured on MS media fortified with NAA produced lower number of shoots compared to control. The highest number of shoots produced, 12.30±0.40 was observed in MS medium supplemented with 1.0 mg/l BAP and 0.5 mg/l NAA. Cultured microtubers also produced roots in all the media tested. The highest root formation (7.04±0.13) was observed when microtubers were cultured on MS medium supplemented with 2.0 mg/l NAA. Higher number of roots was observed in MS media fortified with NAA compared to control and MS media supplemented with BAP alone.

Table 4.5: Mean number of microshoots and root formation from microtubers of *Dioscorea alata* L. cultured on MS media supplemented with different concentrations and combinations of plant growth regulators maintained at 25±1^{0}C with 16 h light and 8 h dark. Thirty replicates were used for each treatment.

MS + Plant Growth Regulators (mg/l)	(%) of microtubers conversion	No. of microshoot per plantlet	No. of root per plantlet
MS Basal	66.67±0.09^{a}	4.70 ±0.18^{h}	1.70±0.11^{hi}
MS + 0.5 mg/l BAP	73.33±0.08^{a}	8.27±0.25^{ef}	2.14±0.20^{h}
MS+1.0 mg/l BAP	73.33±0.08^{a}	11.45±0.30^{b}	1.59±0.13^{i}
MS+1.5 mg/l BAP	73.33±0.08^{a}	9.55±0.13^{d}	1.86±0.17^{hi}
MS+2.0 mg/l BAP	73.33±0.08^{a}	8.82±0.16^{e}	2.05±0.15^{hi}
MS+0.5 mg/l NAA	66.67±0.09^{a}	4.60 ±0.11^{h}	4.45±0.11^{f}
MS+1.0 mg/l NAA	66.67±0.09^{a}	3.45±0.11^{i}	5.40±0.11^{cd}
MS+1.5 mg/l NAA	76.67±0.08^{a}	3.48±0.16^{i}	5.87±0.14^{bc}
MS+2.0 mg/l NAA	80.00±0.07^{a}	2.63±0.10^{j}	7.04±0.13^{a}
MS + 1.0 mg/l BAP + 0.5 mg/l NAA	80.00±0.07^{a}	12.30±0.40^{a}	4.71±0.16^{ef}
MS + 1.0 mg/l BAP + 1.0 mg/l NAA	80.00±0.07^{a}	12.13±0.23^{a}	4.96±0.19^{de}
MS + 1.0 mg/l BAP + 1.5 mg/l NAA	73.33±0.08^{a}	10.91±0.17^{b}	5.41±0.11^{cd}
MS + 1.0 mg/l BAP + 2.0 mg/l NAA	80.00±0.07^{a}	10.21±0.15^{c}	7.00±0.16^{a}
MS+2.0 mg/l NAA +0.5 mg/l BAP	76.67±0.08^{a}	6.52±0.14^{g}	4.00±0.18^{g}
MS+2.0 mg/l NAA +1.5 mg/l BAP	73.33±0.08^{a}	8.60±0.14^{e}	6.05±0.20^{b}
MS+2.0 mg/l NAA +2.0 mg/l BAP	76.67±0.08^{a}	7.83±0.17^{f}	4.48±0.23^{f}

Mean values with different letters within a column are significantly different at p=0.05, by one way ANOVA and Duncan's Multiple Range Test (DMRT).

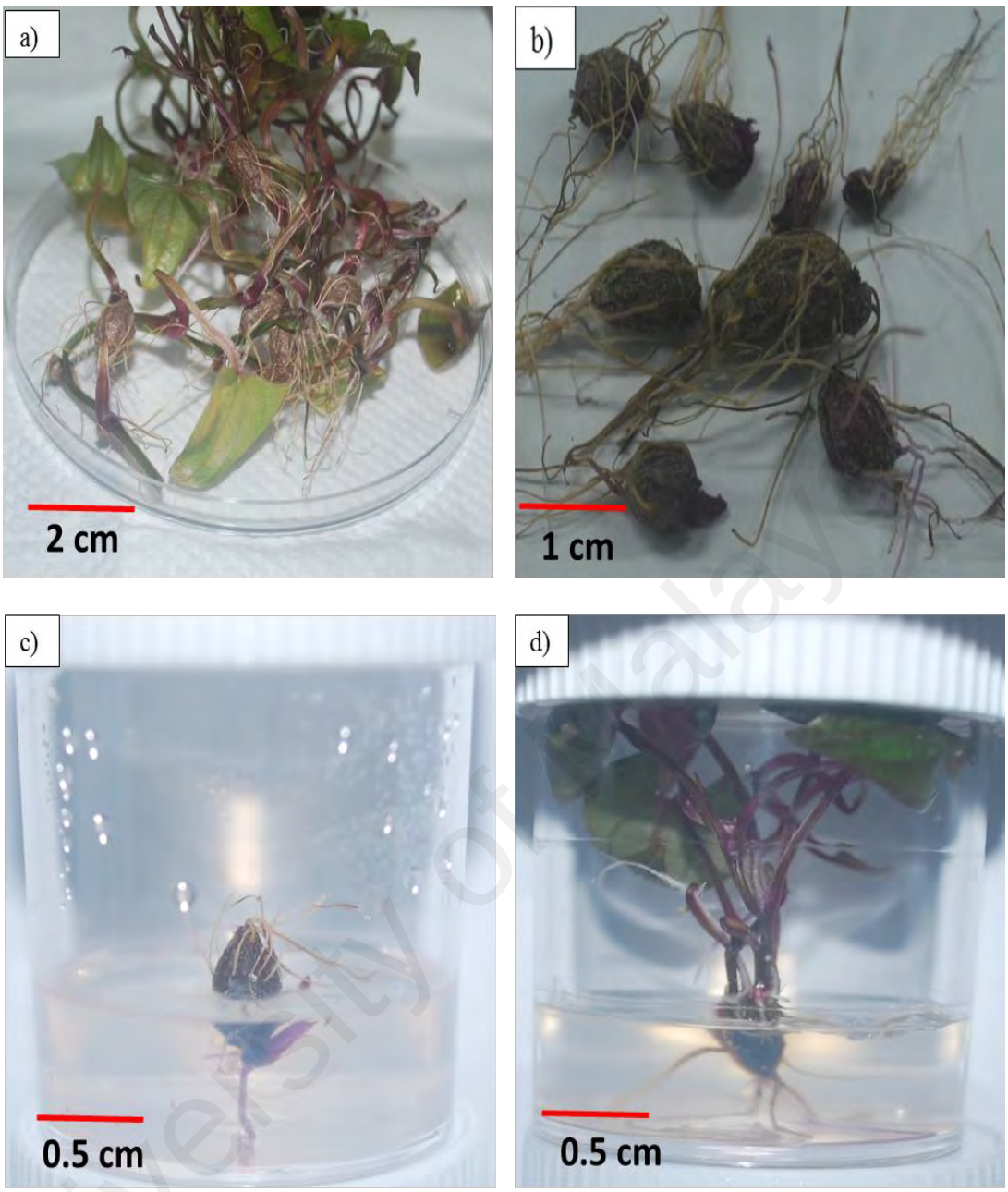


Figure 4.5: a) Microtubers formation on plantlet cultured on MS medium supplemented with 1.0 mg/l NAA and 1.0 mg/l BAP, b) Microtubers harvested from complete plantlet, c) Conversion of microtuber after 1 month in culture and d) Complete plantlet with shoots and roots regenerated from microtubers.

4.2 Coloured callus induction from various explants of *Dioscorea alata* L.

Callus induction from leaf, stem and node explants of *Dioscorea alata* L. had been successfully achieved. Callus initiation was observed from the cut edges of the explants then callus extension to progressively cover the whole explants. Callus with nodular, friable, soft, wet looking surface and purplish white in colour was obtained from all types of explants used. Soft fine purplish white callus was observed from explants cultured on MS media supplemented with Kin.

Callus formation from leaf explant was detected after 4 weeks of culture on the callus induction media. Callus induction occurred from explant wounding and the callus proliferation was fast. After 3 months the whole surface of explant was covered with purple coloured callus (Figure 4.6). From observation, leaf explant managed to produce callus in all media tested except control. The lowest frequency (16.67±0.07) of explant produced callus was observed on MS medium supplemented with 2.0 mg/l Kin (Table 4.6). From all the media tested, MS medium supplemented with 2.0 mg/l 2,4-D and 1.0 mg/l BAP was the best medium for callus formation. The leaf explant gave 93.33±0.05 percent callus formation (Table 4.6).

The initiation of callus formation from stem (internode) explant was observed after 5 weeks of culture on the callus induction media. The callus formation was observed starting from cut edge of the explant (Figure 4.7) and within 3 months, the whole explants were covered with purple coloured callus. Table 4.7 shows the results obtained for callus induction from stem explant. The results revealed that stem explants successfully produced callus in all media tested except for control medium. The lowest percentage of explant produced callus, (10.00±0.06) was observed in MS medium supplemented with

2.0 mg/l Kin, while the highest percentage of explant produced callus (96.67±0.00) was obtained in MS medium supplemented with 2.0 mg/l 2,4-D and 1.0 mg/l BAP (Table 4.7).

Initiation of callus from node explant was observed after 2 weeks of the culture when the lateral buds of the node segment swelled and formed callus (Figure 4.8). A small clump of purple whitish undifferentiated cells could be observed. After 3 months, the callus proliferated 3 to 4 fold in dimension (Figure 4.9). Formation of callus from node explant was detected in all media tested except for control which gave low percentage of callus formation, only 20.00±0.07 % (Table 4.8). Higher percentage of explant produced callus, (96.67±0.03 %) was observed in MS medium supplemented with 2.0 mg/l 2,4-D and 1.0 mg/l BAP (Table 4.8).

MS either solid or liquid media also influence the frequency of explant produced callus in *Dioscorea alata*. The highest explant with callus formation was observed in MS liquid media. All (100.00±0.00 %) of the cultured explants (leaves, nodes and stems) managed to produce callus (Table 4.9). Activated charcoal added to the media reduced the percentage of callus formation. For leaf explant, MS media supplemented with 2.0 mg/l 2,4-D and 1.0 mg/l BAP was the best media for callus formation with 93.33±0.05 % explants produced callus. However, in the same media with additional of 0.1 mg/l activated charcoal, only 10.00±0.06 % of leaf explant managed to produce callus (Table 4.10). Explants producing callus was decreased to 80 % was observed in all the media tested with all types of explant used.

Myo-inositol is mostly applied in the media as it is believed to enhance the plant regeneration, although the basis for its requirement is unknown. Table 4.11 shows the effect of additional myo-inositol in the culture media in terms of the percentage of

explants producing coloured callus. Leaf, stem and node explants successfully produced callus in all media tested. For leaf and stem explants, the percentage of explants produced callus were the same in control and in callus induction media supplemented with 0.1 mg/l myo-inositol or 0.5 mg/l myo-inositol. The results indicated that supplementary myo-inositol did not affect much on the percentage of explants producing callus for leaf and stem explants. However, early initiation of callus was observed as the explants were cultured on the media added with myo-inositol and the callus grown vigorously. For node explant, additional of 0.1 mg/l myo-inositol in the callus induction media gave the same result as control (90.00±0.06 %). Nevertheless, additional of 0.5 mg/l myo-inositol managed to increase the percentage of explant producing callus to 96.67±0.05 % in MS medium supplemented with 2.0 mg/l 2,4-D and 93.33±0.05 % in MS medium supplemented with 2.0 mg/l NAA.

Light has been utilized to improve the efficiency of tissue culture system for many plant species. Determination of the sensitivity of explants produce callus to light quality and quantity may result in better utilization of the light environment as a regulator in coloured callus production. In this study, percentage of callus formation was found to be independent from the various time of light exposure. Explants managed to produce callus in all treatments (Table 4.12), no significant difference observed on average callus formation frequency but explant cultured under light condition gave slightly larger average size in callus than those cultured under dark condition. Nevertheless, four different colours of callus was observed when explants exposed to four different photoperiods (Figure 4.10).

Table 4.6: Callus induction from leaf explants of *Dioscorea alata* L. cultured on MS supplemented with various concentrations of plant growth regulators maintained at 25±1 ºC under 16 hours light and 8 hours dark. Thirty replicates were used in each treatment. Data were recorded after 3 months of culture.

MS + Plant Growth Regulators (mg/l)	Percentage of leaf explant produced callus (mean ± SE)	Observations
MS Basal	0.00±0.00^{f}	No callus formation was observed.
MS+0.5 2,4-D	60.00±0.09^{de}	Explant swollen and red purplish caloured callus was observed.
MS+1.0 2,4-D	66.67±0.09^{cd}	Explant swollen and red purplish caloured callus was observed.
MS+1.5 2,4-D	83.33±0.07^{ab}	Explant swollen and red purplish caloured callus was observed.
MS+2.0 2,4-D	93.33±0.05^{a}	Explant swollen and red purplish caloured callus was observed.
MS+0.5 NAA	46.67±0.09^{de}	Explant swollen and red purplish caloured callus was observed.
MS+1.0 NAA	56.67±0.09^{de}	Explant swollen and red purplish caloured callus was observed.
MS+1.5 NAA	73.33±0.08^{bc}	Explant swollen and red purplish caloured callus was observed
MS+2.0 NAA	90.00±0.06^{a}	Explant swollen and red purplish caloured callus was observed
MS+0.5 KIN	23.33±0.08^{f}	Explant swollen and soft fine purplish caloured callus was observed.
MS+1.0 KIN	43.33±0.09^{e}	Explant swollen and soft fine purplish caloured callus was observed.
MS+1.5 KIN	50.00±0.09^{de}	Explant swollen and soft fine purplish caloured callus was observed.
MS+2.0 KIN	16.67±0.07^{f}	Explant swollen and soft fine purplish caloured callus was observed.
MS+2.0 2,4-D+1.0 BAP	93.33±0.05^{a}	Explant swollen and red purplish caloured callus was observed
MS+2.0NAA +1.0 BAP	83.33±0.07^{ab}	Explant swollen and red purplish caloured callus was observed

Means followed by the same letter are not significantly different at the 0.05 level of confidence by one way ANOVA.

Table 4.7: Callus induction from stem explants of *Dioscorea alata* L. cultured on MS supplemented with various concentrations of plant growth regulators maintained at 25±1 ºC under 16 hours light and 8 hours dark. Thirty replicates were used in each treatment. Data were recorded after 3 months of culture.

MS + Plant Growth Regulators (mg/l)	Percentage of stem explant produced callus (mean ± SE)	Observations
MS Basal	0.00±0.00^{f}	Explant swollen and red purplish caloured callus was observed.
MS+0.5 2,4-D	63.33±0.09^{de}	Explant swollen and red purplish caloured callus was observed.
MS+1.0 2,4-D	73.33±0.08^{bcd}	Explant swollen and red purplish caloured callus was observed.
MS+1.5 2,4-D	93.33±0.05^{ab}	Explant swollen and red purplish caloured callus was observed
MS+2.0 2,4-D	93.33±0.05^{ab}	Explant swollen and red purplish caloured callus was observed.
MS+0.5 NAA	56.67±0.09^{de}	Explant swollen and red purplish caloured callus was observed.
MS+1.0 NAA	63.33±0.09 ^{de}	Explant swollen and red purplish caloured callus was observed.
MS+1.5 NAA	83.33±0.07^{bc}	Explant swollen and red purplish caloured callus was observed.
MS+2.0 NAA	86.67±0.06^{ab}	Explant swollen and red purplish caloured callus was observed.
MS+0.5 KIN	20.00±0.07^{f}	Explant swollen and soft fine purplish caloured callus was observed.
MS+1.0 KIN	43.33±0.09^{e}	Explant swollen and soft fine purplish caloured callus was observed.
MS+1.5 KIN	46.67±0.09^{e}	Explant swollen and soft fine purplish caloured callus was observed.
MS+2.0 KIN	10.00±0.06^{f}	Explant swollen and soft fine purplish caloured callus was observed.
MS+2.0 2,4-D+1.0 BAP	96.67±0.03^{a}	Explant swollen and red purplish caloured callus was observed.
MS+2.0 NAA+1.0 BAP	83.33±0.07^{abc}	Explant swollen and red purplish caloured callus was observed.

Means followed by the same letter are not significantly different at the 0.05 level of confidence by one way ANOVA.

Table 4.8: Callus induction from node explants of *Dioscorea alata* L. cultured on MS supplemented with various concentrations of plant growth regulators maintained at 25±1 ºC under 16 hours light and 8 hours dark. Thirty replicates were used in each treatment. Data were recorded after 3 months of culture.

MS + Plant Growth Regulators (mg/l)	Percentage of node explant produced callus (mean ± SE)	Observations
MS Basal	0.00±0.00^{g}	Red purplish caloured callus was observed.
MS+0.5 2,4-D	60.00±0.09^{cd}	Red purplish caloured callus was observed.
MS+1.0 2,4-D	76.67±0.08^{abc}	Red purplish caloured callus was observed.
MS+1.5 2,4-D	90.00±0.06^{a}	Red purplish caloured callus was observed.
MS+2.0 2,4-D	90.00±0.06^{a}	Red purplish caloured callus was observed.
MS+0.5 NAA	40.00±0.09^{ef}	Red purplish caloured callus was observed.
MS+1.0 NAA	66.67±0.09^{bc}	Red purplish caloured callus was observed.
MS+1.5 NAA	83.33±0.07^{ab}	Red purplish caloured callus was observed.
MS+2.0 NAA	90.00±0.09^{a}	Red purplish caloured callus was observed.
MS+0.5 KIN	23.33±0.08^{f}	Red purplish caloured callus was observed.
MS+1.0 KIN	43.33±0.09^{de}	Red purplish caloured callus was observed.
MS+1.5 KIN	40.00±0.09^{ef}	Red purplish caloured callus was observed.
MS+2.0 KIN	20.00±0.07^{g}	Red purplish caloured callus was observed.
MS+2.02,4-D+1.0BAP	96.67±0.03^{a}	Red purplish caloured callus was observed.
MS+2.0NAA+1.0 BAP	86.67±0.06^{ab}	Red purplish caloured callus was observed.

Means followed by the same letter are not significantly different at the 0.05 level of confidence by one way ANOVA.

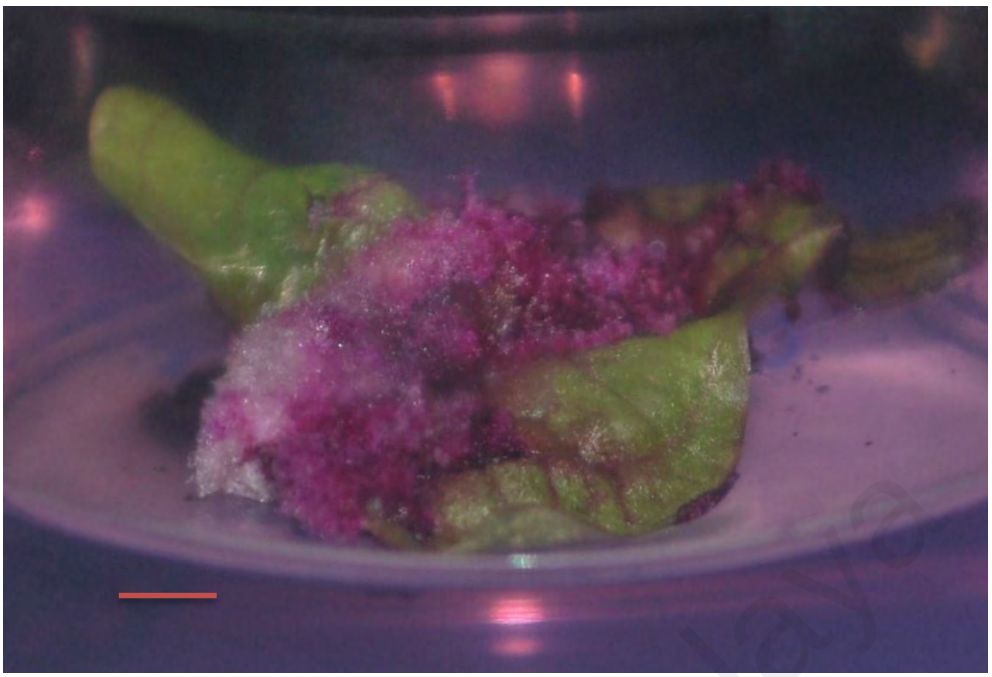


Figure 4.6: Callus derived from leaf explant cultured on MS medium supplemented with 2.0 mg/l 2,4-D and 1.0 mg/l BAP. Bars = 1 mm.

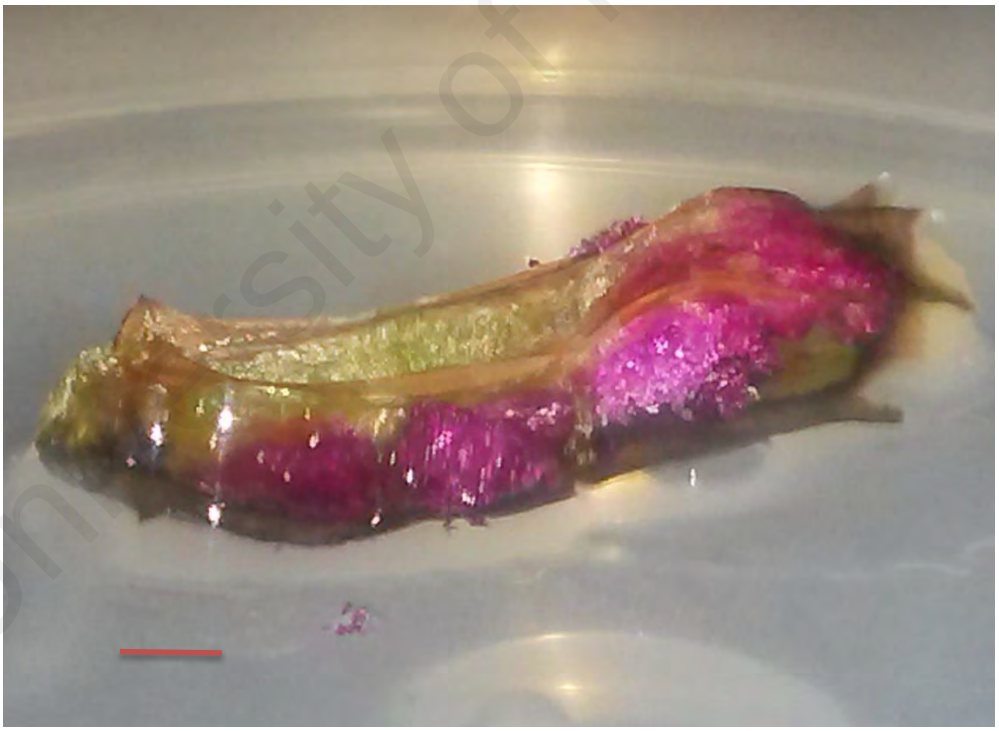


Figure 4.7: Callus derived from stem (internode) explant cultured on MS medium supplemented with 2.0 mg/l 2,4-D and 1.0 mg/l BAP. Bars = 1 mm.

Figure 4.8: Swollen of lateral bud in node explant cultured on MS medium supplemented with 2.0 mg/l 2,4-D and 1.0 mg/l BAP. Bars = 1 mm.

Figure 4.9: Callus derived from node explant cultured on MS medium supplemented with 2.0 mg/l 2,4-D and 1.0 mg/l BAP. Bars = 1 mm.

Table 4.9: Callus induction from various explants of *Dioscorea alata* L cultured on solid and liquid MS media supplemented with 2.0 mg/l 2,4-D added with 0.5 g/l Myo-inositol maintained at 25±1^{0}C with 16 hours light and 8 hours dark. Thirty replicates were used in each treatment. Data were recorded after 3 months of culture.

Explant	**Percentage of explant produced callus (mean ± SE)**		**Observations**
	Solid Media	Liquid media	
Leaf	83.33±0.07^b	100.00±0.00^a	Solid: Explant become swollen and purple friable callus formed. Liquid: Explant become swollen 10 times. Purple, friable, and soft (watery) callus was formed.
Stem	83.33±0.07^b	100.00±0.00^a	Solid: Explant become swollen and purple friable callus formed. Liquid: Explant become swollen. Purple, friable, and soft (watery) callus was formed.
Node	86.67±0.06^b	100.00±0.00^a	Solid: Explant become swollen and purple friable callus formed. Liquid: Explant become swollen 2 times. Purple, friable, and soft (watery) callus was formed.

Means followed by the same letter are not significantly different at the 0.05 level of confidence by one way ANOVA.

Table 4.10: Effects of activated charcoal on callus induction from various explants of *Dioscorea alata* L. cultured on MS media supplemented with various concentrations of plant growth regulators maintained at 25±1 ºC under 16 hours light and 8 hours dark. Thirty replicates were used in each treatment. Data were recorded after 3 months of culture.

Explant	**Percentage of explant produced callus (mean ± SE)**		
	Media	- charcoal	+ Charcoal
Leaf	MS + 2.0 mg/l 2,4-D	93.33±0.05*	06.67±0.05*
	MS +2.0 mg/l 2,4-D +1.0 mg/l BAP	93.33±0.05*	10.00±0.06*
	MS + 2.0 mg/l NAA	90.00±0.06*	13.33±0.06*
	MS + 2.0 mg/l NAA + 1.0 mg/l BAP	83.33±0.07*	06.67±0.05*
	MS + 1.0 mg/l TDZ	90.00±0.06*	03.33±0.03*
	MS + 2.0 mg/l TDZ	90.00±0.06*	10.00±0.06*
Stem	MS + 2.0 mg/l 2,4-D	93.33±0.05*	10.00±0.06*
	MS + 2.0 mg/l 2,4-D + 1.0 mg/l BAP	96.67±0.03*	10.00±0.06*
	MS + 2.0 mg/l NAA	90.00±0.06*	13.33±0.06*
	MS + 2.0 mg/l NAA + 1.0 mg/l BAP	83.33±0.07*	06.67±0.05*
	MS + 1.0 mg/l TDZ	83.33±0.07*	03.33±0.03*
	MS + 2.0 mg/l TDZ	86.67±0.03*	10.00±0.06*
Node	MS + 2.0 mg/l 2,4-D	90.00±0.06*	10.00±0.06*
	MS + 2.0 mg/l 2,4-D + 1.0 mg/l BAP	96.67±0.03*	13.33±0.06*
	MS + 2.0 mg/l NAA	90.00±0.06*	16.67±0.07*
	MS + 2.0 mg/l NAA + 1.0 mg/l BAP	86.67±0.06*	10.00±0.06*
	MS + 1.0 mg/l TDZ	86.67±0.06*	03.33±0.03*
	MS + 2.0 mg/l TDZ	86.67±0.06*	10.00±0.06*

*Significant difference at p=0.05 (Independent Samples T-test).

Table 4.11: Effects of myo-inositol on callus induction from various explants of *Dioscorea alata* L. cultured on MS medium supplemented with 2.0 mg/l 2,4-D and MS medium supplemented with 2.0 mg/l NAA maintained at 25±1 ^{0}C with 16 hours light and 8 hours dark. Thirty replicates were used for each treatment. Data were recorded after 3 months of culture.

Explant	**Media**	**Percentage of explant produced callus (mean ± SE)**		
		- Myo-inositol	**+ Myo inositol**	
		(control)	**0.1 mg/l**	**0.5 mg/l**
Leaf	MS + 2.0 mg/l 2,4-D	93.33±0.05^a	93.33±0.05^a	93.33±0.05^a
	MS + 2.0 mg/l NAA	90.00±0.06^a	90.00±0.06^a	90.00±0.06^a
Stem	MS + 2.0 mg/l 2,4-D	93.33±0.05^{ab}	93.33±0.05^a	93.33±0.05^a
	MS + 2.0 mg/l NAA	90.00±0.06^a	90.00±0.06^a	90.00±0.06^a
Node	MS + 2.0 mg/l 2,4-D	90.00±0.06^a	90.00±0.06^a	96.67±0.05^a
	MS + 2.0 mg/l NAA	90.00±0.06^a	90.00±0.06^a	93.33±0.05^a

Means followed by the same letter are not significantly different at the 0.05 level of confidence by one way ANOVA.

Table 4.12: Callus induction from various explants of *Dioscorea alata* L. cultured on MS medium supplemented with 2.0 mg/l 2,4-D and added with 0.5 mg/l myo-inositol maintained under different photoperiod.

Photoperiod/Light exposure	Explant	**Percentage of explant produced callus (mean ± SE)**	**Observations**
24 Light /0 Dark	Leaf	86.67±0.06^{ab}	Explant swollen and dark purple caloured callus was observed after 8 days in cultured.
	Stem	86.67±0.06^{ab}	
	Node	90.00±0.06^a	
16 Light /8 Dark	Leaf	86.67±0.06^{ab}	Explant swollen and purple caloured callus formed after 8 days in cultured.
	Stem	86.67±0.06^{ab}	
	Node	90.00±0.06^a	
12 Light /12 Dark	Leaf	83.33±0.07^{ab}	Explant swollen and red purplish caloured callus was observed after 10 days in cultured.
	Stem	83.33±0.07^{ab}	
	Node	86.67±0.06^{ab}	
0 Light /24 Dark	Leaf	86.67±0.06^{ab}	Explant swollen and crystal white purplish formed after 14 days in cultured.
	Stem	90.00±0.06^a	
	Node	93.33±0.07^{ab}	

Means followed by the same letter are not significantly different at the 0.05 level of confidence by one way ANOVA.

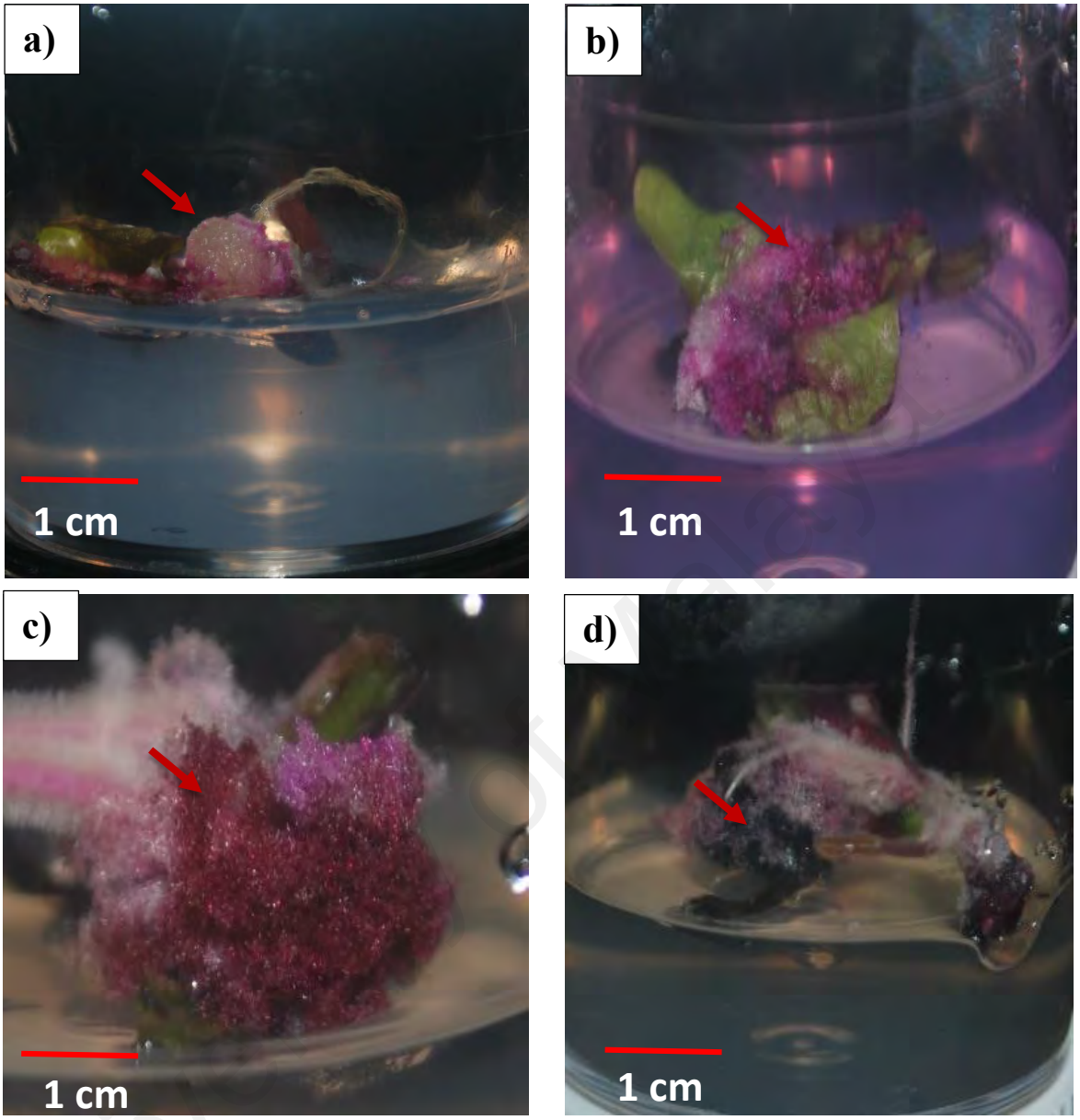


Figure 4.10: Different colours of callus produced from explants cultured on MS medium supplemented with 2 mg/l 2,4-D. a) whitish purple caloured callus observed in the dark b) purple coloured callus observed under 8 hours light, c) red purplish coloured callus observed under 16 hours light and d) dark purple coloured callus observed under 24 hours light.

4.3 Somatic embryogenesis and indirect regeneration of *Dioscorea alata* L.

4.3.1 Induction of embryogenic callus

After 2 weeks in culture, node explants started to form callus around the cut edges of the explants. Callus obtained was purplish white, nodular, soft and watery in structure (Figure 4.11). Figure 4.12 shows, embryogenic callus observed under Dino-Lite USB Digital Microscope. Many globules or Proembryogenic masses (PEMs) were observed. Formation of embryogenic callus from node explants cultured in MS media supplemented with various concentrations of NAA and 2,4-D was presented in Table 4.13. Node explants successfully produced callus in all media tested except in MS medium without plant growth regulators. No formation of callus was observed in MS basal medium. The lowest percentage of explant producing callus, (40.00±0.09 %) was observed in MS medium supplemented with 0.5 mg/l NAA. The highest percentage of explants producing callus, (93.33±0.46 %) was observed on MS medium supplemented with 2.0 mg/l 2,4-D and 2.5 mg/l 2,4-D.

Node explants cultured onto MS media supplemented with 2,4-D at all concentrations showed better response compared to node explants cultured onto MS media fortified with NAA. In all concentrations tested, higher percentage of explants producing callus were observed with the presence of 2,4-D. High concentration of auxin (more than 2.5 mg/l NAA and 2,4-D) showed lower percentage of explants which can produce callus. The percentage of explants produced callus (90.00±0.56 %) was observe in MS medium supplemented with 3.0 mg/l 2,4-D compared to 93.33±0.46 in 2.5 mg/l 2,4-D. Same observation was obtained in MS media supplemented with NAA. Decreasing of more than 3.0 percent of callusing was detected in all media tested.

Table 4.13: Induction of embryogenic callus from node explants cultured on MS solid media supplemented with different concentrations of NAA.

MS + Plant Growth Regulators (mg/l)	Callus formation (%)	Callus characteristics
MS Basal	0.00 ± 0.00[e]	No callus formation
0.5 mg/l NAA	40.00±0.09[d]	Soft watery in structure and whitish purple in colour.
1.0 mg/l NAA	70.00±0.09[bc]	Soft watery in structure and whitish purple in colour.
1.5 mg/l NAA	83.33±0.07[ab]	Soft watery in structure and whitish purple in colour.
2.0 mg/l NAA	90.00±0.06[ab]	Soft watery in structure and whitish purple in colour.
2.5 mg/l NAA	90.00±0.06[ab]	Soft watery in structure and whitish purple in colour.
3.0 mg/l NAA	86.67±0.03[ab]	Soft watery in structure and whitish purple in colour.
0.5 mg/l 2,4-D	60.00±0.09[c]	Soft watery in structure and whitish purple in colour.
1.0 mg/l 2,4-D	80.00±0.74[ab]	Soft watery in structure and whitish purple in colour.
1.5 mg/l 2,4-D	90.00±0.56[ab]	Soft watery in structure and whitish purple in colour.
2.0 mg/l 2,4-D	93.33±0.46[a]	Soft watery in structure and whitish purple in colour.
2.5 mg/l 2,4-D	93.33±0.46[a]	Soft watery in structure and whitish purple in colour.
3.0 mg/l 2,4-D	90.00±0.56[ab]	Soft watery in structure and whitish purple in colour.

Means followed by the same letter are not significantly different at the 0.05 level of confidence by one way ANOVA

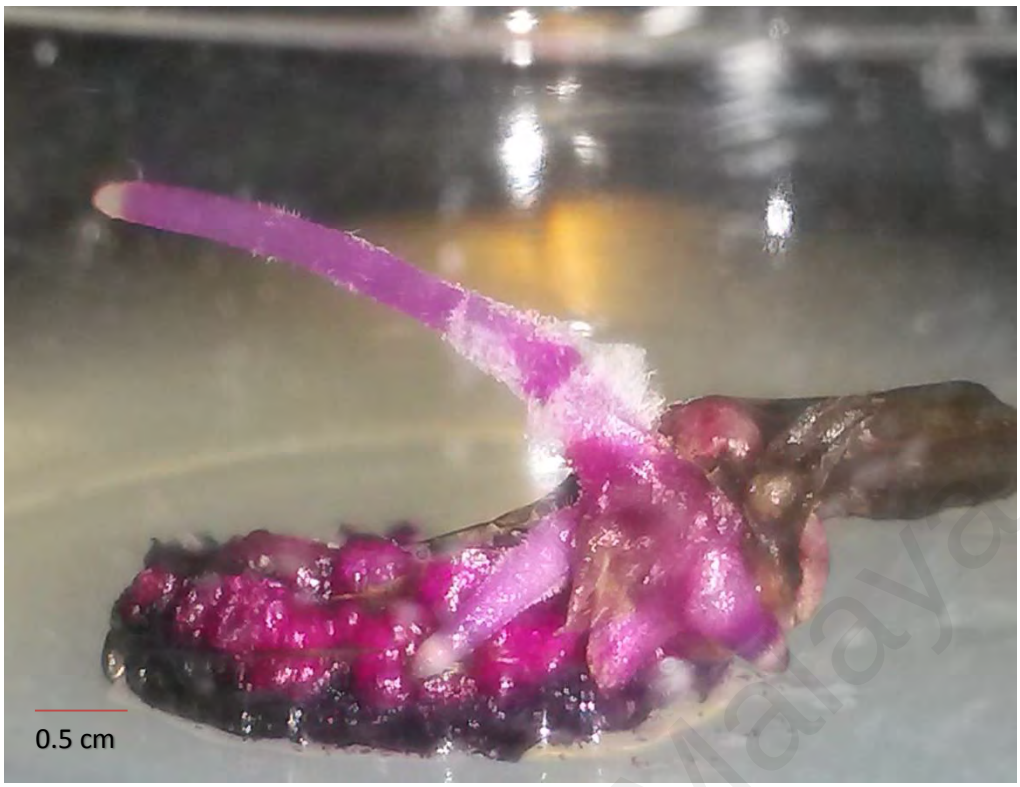


Figure 4.11: Embryogenic callus derived from node explant of *Dioscorea alata* L. cultured on MS medium supplemented with 2.0 mg/l 2,4-D.

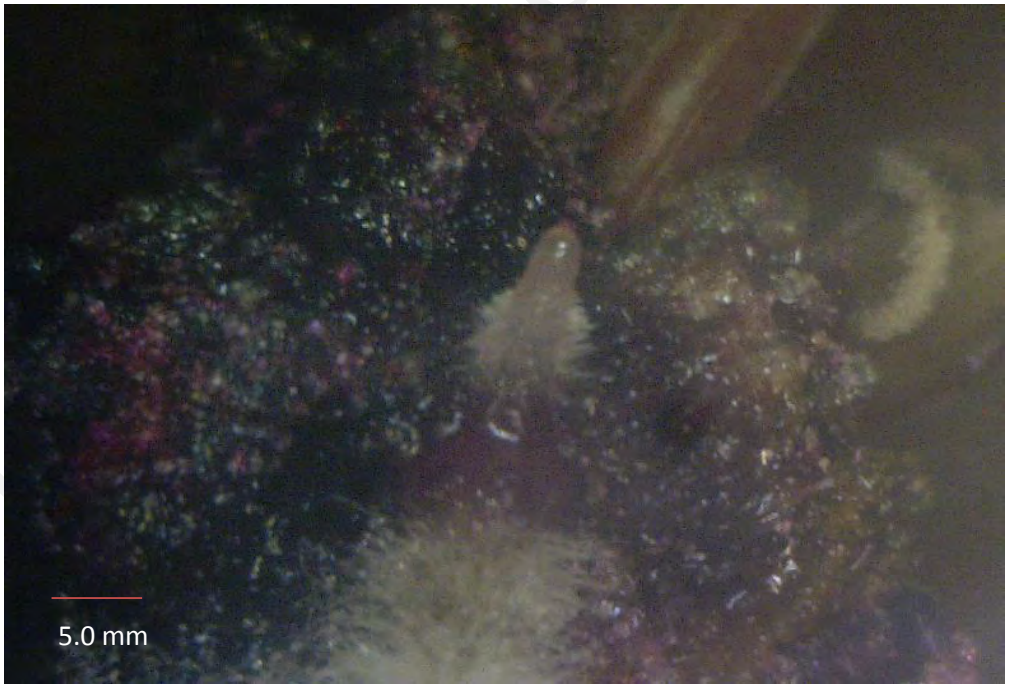


Figure 4.12: Emryogenic callus derived from node explant of *Dioscorea alata* L. observed under Dino-light microscope

4.3.2 Identification of embryogenic callus

Callus obtained from node explants were than identified whether embryogenic or non embryogenic in nature using double staining method and observed under light microscope. Embryogenic callus cells were stained red with acetocarmine (Figure 4.13a and 4.13b) and non-embryogenic callus cells were stained blue with Evan's blue (Figure 4.13c).

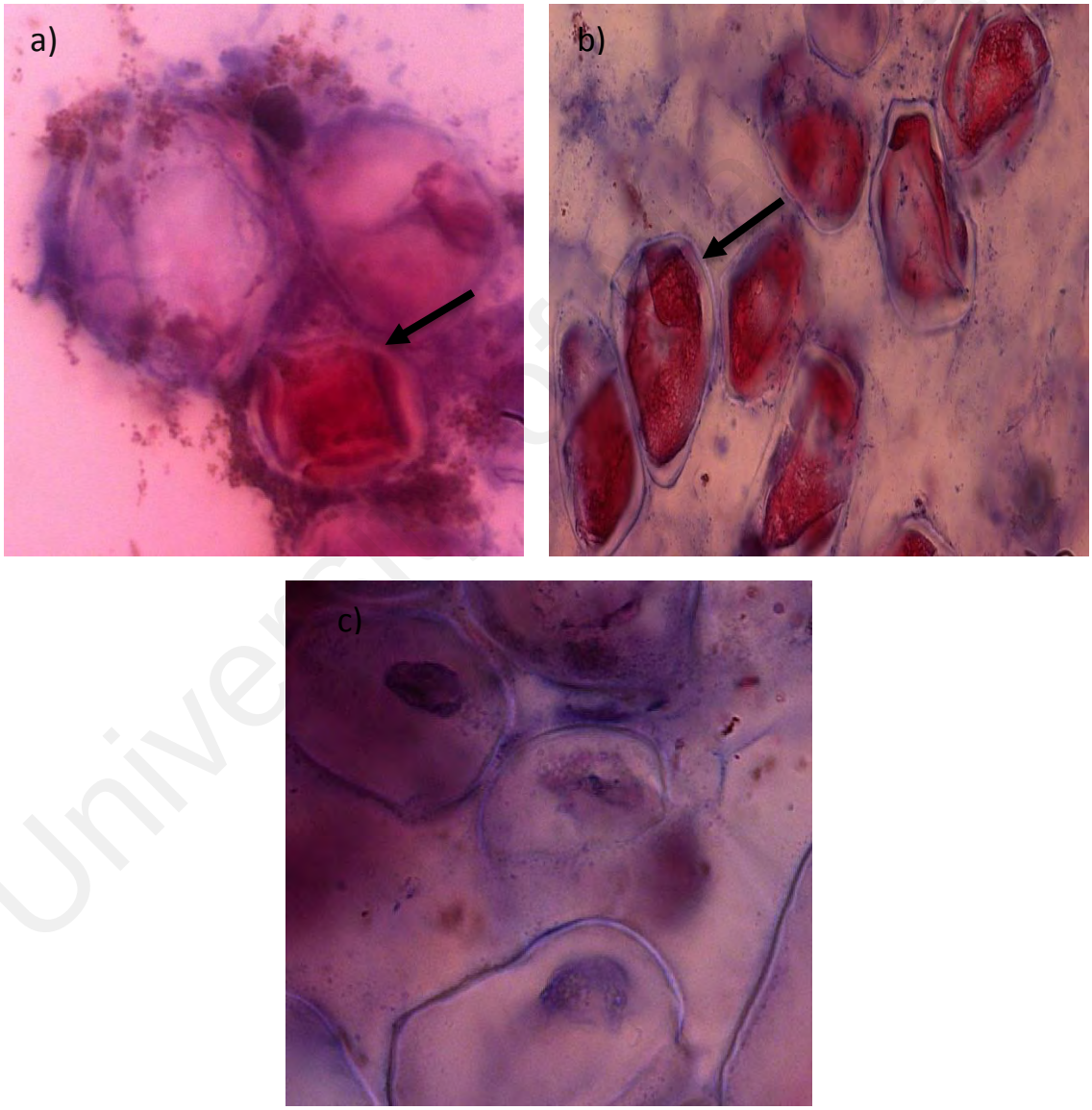


Figure 4.13: a) Embryogenic callus cells stained red with acetocarmine, b) Large nuclei of embryogenic callus, c) Non-embryogenic callus cells stained blue with Evan's blue. Observed under a light microscope. Magnification 50X.

4.3.3 Somatic embryo development

In order to enhance the formation and production of mature development stages of somatic embryos, embryogenic callus (Figure 4.11) was transferred or subcultured onto somatic embryo induction media consist of MS solid and liquid media supplemented with different combinations and concentrations of BAP, NAA and 2,4-D. The mean number of somatic embryos formation in solid and liquid media were presented in Table 4.14. Embryogenic callus after being transferred to MS plant growth regulators free medium and MS supplemented with only BAP did not produced any somatic embryo. Browning of embryogenic callus was detected and after some time in culture, the callus became necrotic and death of tissues was observed. However, on the media supplemented with BAP in combination with 2,4-D, smooth round purplish structure with a diameter 3 to 5 mm occurred on the surface of embryogenic callus within 3 months. Figure 4.14 and 4.15 showed somatic embryo observed on MS medium supplemented with 1.0 mg/l BAP and 2.0 mg/l 2,4-D.

Embryogenic callus was also successfully developed into somatic embryos on MS supplemented with NAA and 2,4-D applied singly. The media supplemented with 2,4-D showed high mean number of somatic embryos formation in all media tested with the same concentration. For example, in media supplemented with 2.0 mg/l 2,4-D, 22.43±0.18 mean number of somatic embryo formation in liquid media and 19.43±0.25 mean number of somatic embryos on solid media, while in media supplemented with 2.0 mg/l NAA, 20.10±0.43 of somatic embryo formation in liquid media and 16.57 ±0.17 on solid media. MS medium supplemented with 0.5 mg/l NAA was less favourable for somatic embryo induction. Only 9.23±0.60 somatic embryos was observed in liquid media and 7.00±0.511 on solid media.

MS media supplemented with 2,4-D in combination with BAP promoted somatic embryogenesis effectively compared to NAA applied singly, and 2,4-D applied singly (Table 1.14). The highest frequencies of somatic embryogenesis occurred on MS medium supplemented with 2,4-D at concentration of 2.0 mg/l combined with 1.0 mg/l BAP, where an average of 23.27±0.14 somatic embryos were obtained from 0.5 cm of embryogenic callus from liquid media and 21.13±0.27 somatic embryos were formed on solid media.

Liquid media was the best media for formation of somatic embryos compared to solid media. Higher number of somatic embryos was found in liquid media compared to solid media in every treatment with the same concentrations of supplemented plant growth regulators. For example, in media supplemented with 0.5 mg/l 2,4-D, 14.10±0.15 mean number of somatic embryos were form in liquid media and 16.30±0.71 mean number of somatic embryos on solid media

Somatic embryos at globular stage elongated to form scutellar and subsequently developed further into coleoptilar stages. The developmental stages of somatic embryo of *Dioscorea alata* was clearly observed as shown in Figure 4.16. More than one stages of development was observed in one culture tube.

Table 4.14: Mean number of somatic embryos of *Dioscorea alata* L. cultured in liquid and solid MS media supplemented with different concentrations and combinations of plant growth regulators maintained at 25±1^{0}C with 16 h light and 8 h dark. Thirty replicates were used in each treatment.

MS + Plant Growth Regulators (mg/l)	Number of Somatic Embryo per Explant (1cm x 1cm callus)	
	Liquid media	**Solid media**
1.MS media (control)	0.00±0.00^{l}	0.00±0.00^{j}
2. MS + 0.5 mg/l BAP	0.00±0.00^{l}	0.00±0.00^{j}
3. MS + 1.0 mg/l BAP	0.00±0.00^{l}	0.00±0.00^{j}
4. MS + 1.5 mg/l BAP	0.00±0.00^{l}	0.00±0.00^{j}
5. MS + 2.0 mg/l BAP	0.00±0.00^{l}	0.00±0.00^{j}
6. MS + 0.5 mg/l NAA	7.00±0.511^{k}	9.23±0.60^{i}
7. MS + 1.0 mg/l NAA	10.80±0.28^{j}	12.17±0.43^{h}
8. MS + 1.5 mg/l NAA	14.03±0.47^{i}	16.43±0.57^{g}
9. MS + 2.0 mg/l NAA	16.57 ±0.17^{ef}	20.10±0.43^{de}
10.MS + 2.5 mg/l NAA	15.83±0.08^{h}	19.93±0.43^{f}
11. MS + 3.0 mg/l NAA	15.20±0.17^{h}	16.13±0.56^{g}
12 MS + 0.5 mg/l 2-4,D	14.10±0.15^{i}	16.30±0.71^{g}
13 MS + 1.0 mg/l 2-4,D	16.03±0.15^{fg}	19.93±0.16^{f}
14 MS + 1.5 mg/l 2-4,D	18.07±0.30^{d}	20.60±0.27^{cde}
15 MS + 2.0 mg/l 2-4,D	19.43±0.25^{bc}	22.43±0.18^{b}
16 MS + 2.5 mg/l 2-4,D	19.00±0.14^{c}	22.40±0.18^{b}
17 MS + 3.0 mg/l 2-4,D	17.00±0.19^{e}	20.80±0.22^{cd}
18 MS + 2.0 mg/l 2-4,D + 0.5 mg/l BAP	20.00±0.29^{b}	20.93±0.21^{c}
19 MS + 2.0 mg/l 2-4,D + 1.0 mg/l BAP	21.13±0.27^{a}	23.27±0.14^{a}
20 MS + 2.0 mg/l 2-4,D + 1.5 mg/l BAP	17.09±0.14^{d}	20.47±0.23^{cde}
21 MS + 2.0 mg/l 2-4,D + 2.0 mg/l BAP	18.07±0.14^{d}	20.00±0.14^{e}

Mean values with different letters within a column are significantly different at p=0.05, by oneway ANOVA and Duncan's Multiple Range Test (DMRT).

Figure 4.14: Somatic embryo at globular stage (after 12 weeks).

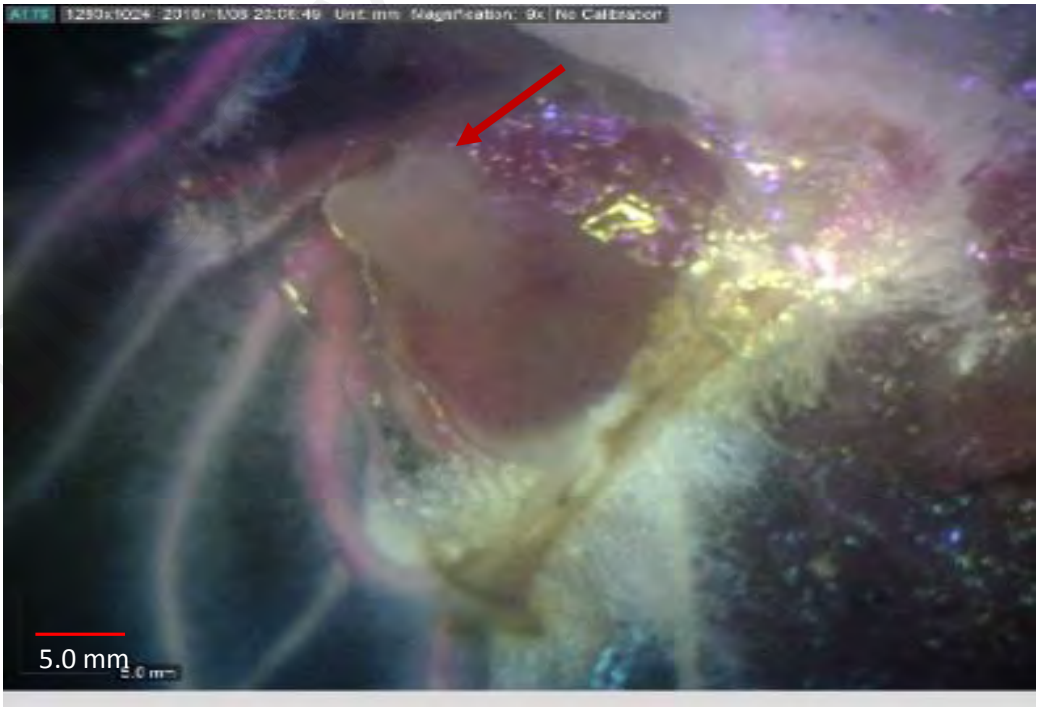


Figure 4.15: Somatic embryo at coleoptilar stage after 13 weeks in culture.

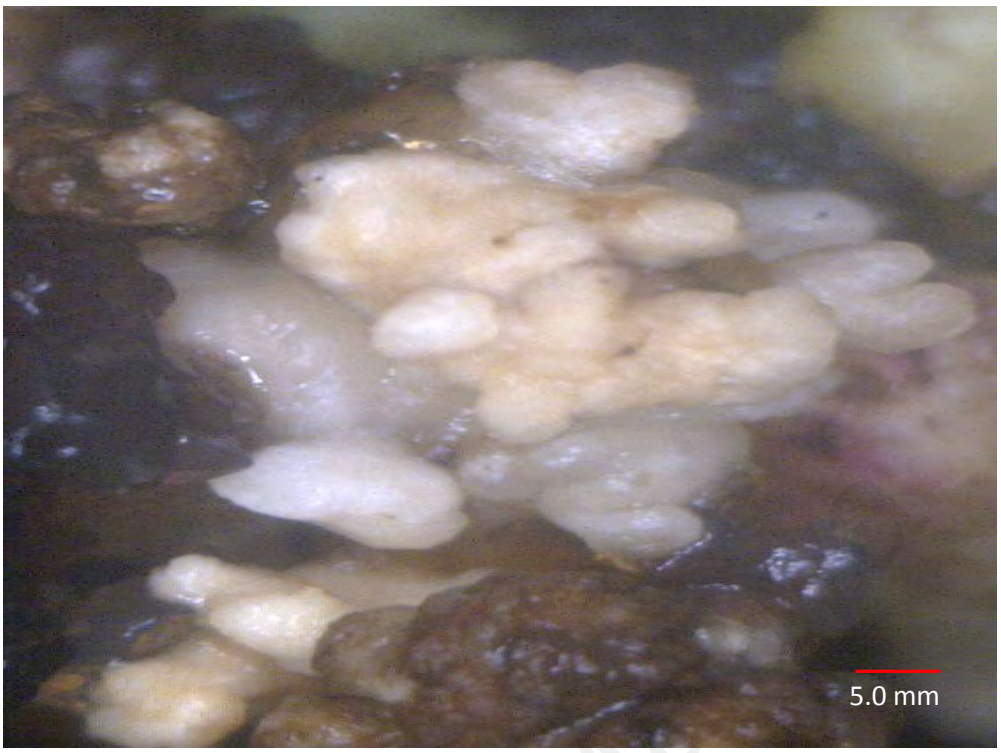


Figure 4.16: Opaque-white somatic embryos developed from embryogenic callus after being transferred onto somatic embryo maturation media.

4.3.4 Somatic embryos germination

After 3 months in culture, immature embryos enlarged into scutellar and coleoptilar stages and subsequently developed into matured embryos with tiny leaves (Figure 4.16). To prompt maturation and germination of embryos, somatic embryos were transferred onto MS media supplemented with different concentrations of BAP and NAA. The embryos successfully germinated after one month being transferred. Hook type of conversion was observed with the formation of cotyledonary collars and tiny leaves emerge from the collars opening (Figure 4.17).

The mean percentage of somatic embryos germination and mean number of shoots and roots formation were presented in Table 4.15. Somatic embryos after being transferred onto MS media supplemented with NAA at all concentrations (0.5- 2.0 mg/l) showed no embryo germination. Embryos did not develop into shoots and roots. In the present study,

the embryos were successfully germinated on MS medium without plant growth regulators (control) but with low percentage of germination, 23.33±0.08 and mean number of shoots formation was 7.00±0.31.

High percentage of embryos germination was observed on MS media supplemented with BAP. The addition of BAP into the media significantly increased the percentage of somatic embryos germination at all concentrations (0.5- 2.0 mg/l BAP). The highest percentage of somatic embryos germination, (60.00±0.09 %) was observed on MS medium supplemented with 1.0 mg/l BAP. The second highest was observed on MS medium supplemented with 0.5 mg/l BAP. Somatic embryos also managed to germinate on MS media fortified with BAP in combination with NAA. However, lower germination rate was observed. The lowest percentage of embryos germination, (10.00±0.06 %) was observed on MS medium supplemented with 1.0 mg/l BAP and 2.0 mg/l NAA.

Formation of shoots (Figure 4.17) depended on the formation of cotyledonary collars. When more somatic embryos developed into cotyledonary collars, more shoots formation were observed. Therefore, addition of BAP into the germination media did not only increase the percentage of embryos germination but also increased the number of regenerated shoots. The highest mean number of shoots produced, 18.00±0.36 was obtained from somatic embryos cultured on MS medium fortified with 1.0 mg/l BAP. The lowest mean number of shoots formation, 3.33±0.88 was detected on MS medium supplemented with 1.0 mg/l BAP and 2.0 mg/l NAA.

Formation of roots (Figure 4.18) was detected from the germination of somatic embryos on MS media supplemented with combinations of BAP and NAA. However, low mean number of root formation was observed. The highest mean number of root

formation, with only 3.50±0.29 was observed on MS medium supplemented with 1.0 mg/l BAP and 1.5 mg/l NAA. No formation of cotyledonary collars was observed for roots formation. Globular stages of somatic embryos become oblong and subsequently formed roots.

Table 4.15: Effects of different concentrations of plant growth regulators on germination of *Dioscorea alata* somatic embryos maintained at 25±1^{0}C with 16 h light and 8 h dark. Thirty replicates were used in each treatment.

Media	% of germination	No of shoots	No of roots
MS Basal (Control)	23.33±0.08^{bcd}	7.00±0.31^{de}	-nil-
MS + 0.5 mg/l BAP	56.67±0.09^{a}	17.06±0.34^{a}	-nil-
MS + 1.0 mg/l BAP	60.00±0.09^{a}	18.00±0.36^{a}	-nil-
MS + 1.5 mg/l BAP	43.33±0.09^{ab}	11.85±0.48^{b}	-nil-
MS + 2.0 mg/l BAP	36.67±0.09^{bc}	9.00±0.43^{c}	-nil-
MS + 0.5 mg/l NAA	0.00 ± 0.00^{e}	-nil-	-nil-
MS + 1.0 mg/l NAA	0.00 ± 0.00^{e}	-nil-	-nil-
MS + 1.5 mg/l NAA	0.00 ± 0.00^{e}	-nil-	-nil-
MS + 2.0 mg/l NAA	0.00 ± 0.00^{e}	-nil-	-nil-
MS + 1.0 mg/l BAP + 0.5 mg/l NAA	30.00±0.09^{bcd}	8.33±0.65^{cd}	3.33±0.44^{a}
MS + 1.0 mg/l BAP + 1.0 mg/l NAA	16.67±0.07^{cde}	5.80±0.37^{ef}	2.60±0.24^{a}
MS + 1.0 mg/l BAP + 1.5 mg/l NAA	13.33±0.06^{de}	4.50±0.65^{fg}	3.50±0.29^{a}
MS + 1.0 mg/l BAP + 2.0 mg/l NAA	10.00±0.06^{de}	3.33±0.88^{g}	3.00±0.58^{a}

Mean values with different letters within a column are significantly different at p=0.05, by one way ANOVA and Duncan's Multiple Range Test (DMRT).

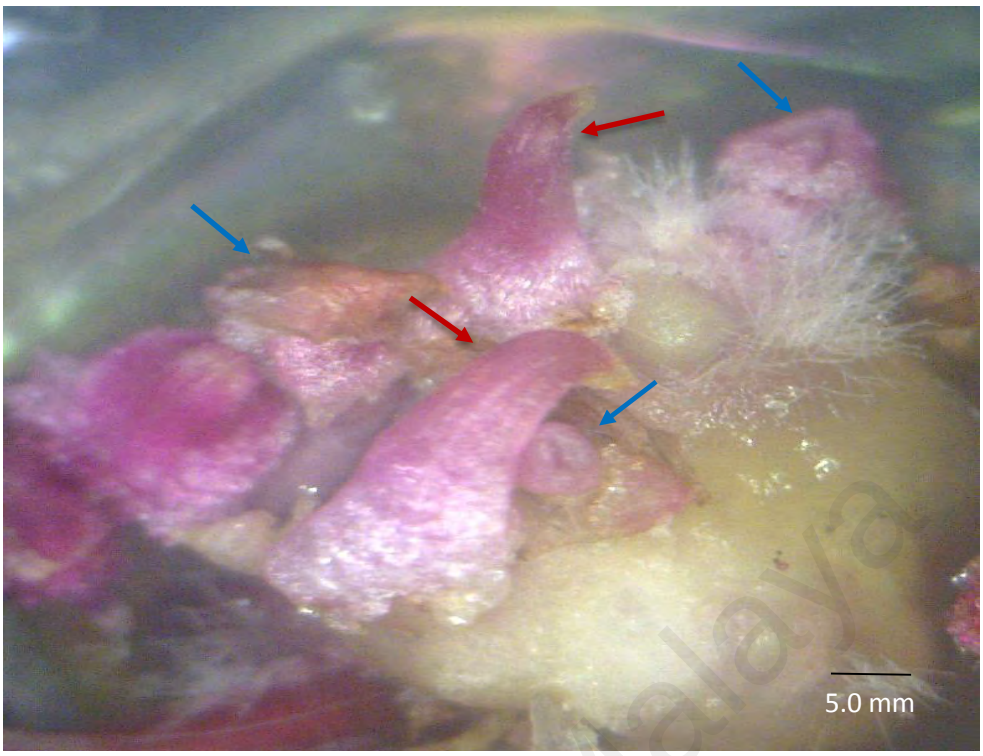


Figure 4.17: Development of microshoots from somatic embryos of *Dioscorea alata* from node explant. Cotyledonary collar and hook type of conversion of *Dioscorea alata*. Red arrows showing shoots formation. Blue arrows showing cotyledonary collars.

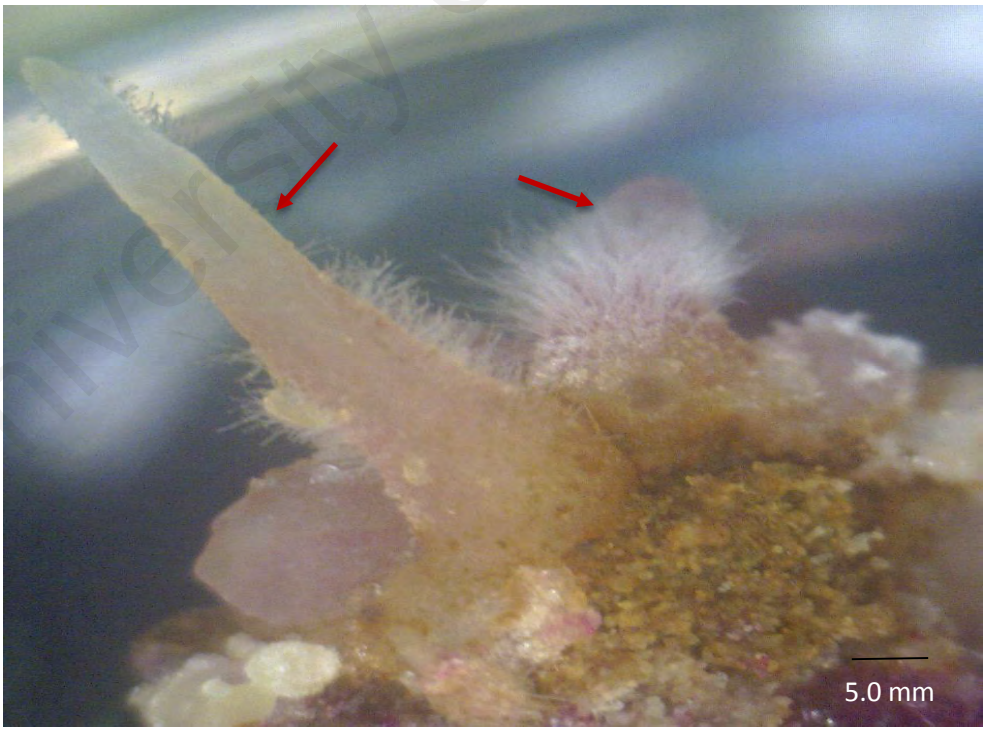


Figure 4.18: Development of roots from somatic embryos of *Dioscorea alata* L. in MS medium supplemented with1.0 mg/l BAP + 1.5 mg/l NAA.

4.4 Synthetic seed studies in *Dioscorea alata* L.

4.4.1 Identification of suitable concentration of Sodium alginate solution and Calcium chloride as an encapsulation matrix

Shoot tips node and stem explants of *Dioscorea alata* were encapsulated with sodium alginate solution and calcium chloride dehydrate to form synthetic seeds (bead form). Figure 4.19 shows formation of synthetic seeds from encapsulated shoot tips, nodes and stem explants of *Dioscorea alata*. Bead formation with ideal texture and uniform in size, isodiametric shape and easy to handle were the important aspect in synthetic seed preparation.

In the present study, propagules were successfully encapsulated in 2.0 to 5.0 % sodium alginate solution and hardened in 25.0 to 100.0 mM calcium chloride dehydrate solution. Results obtained in Table 4.16 showed different concentrations of sodium alginate ($NaC_6H_7O_6$) solution and calcium chloride dehydrate ($CaCl_2.2H_2O$) influenced the bead formation. The beads varied morphologically with respect to texture, shape and transparency with different combinations of sodium alginate solution and calcium chloride. It was found that, 3% sodium alginate was the best encapsulation solution. Propagules encapsulated with 3% sodium alginate solution hardened in 100 µM calcium chloride dehydrate produced beads with firm coats, uniform size with round shape and easy for handling.

No definite shape of beads were formed with low concentration of sodium alginate (2.0%). The beads were difficult to handle and fragile. At higher concentrations of sodium alginate solution (5.0%), the beads were isodiametric in shape but too hard which can cause considerable sprouting delay. However, it was found that encapsulated explants

showed different degree of success on ideal beads produced. Further experiments were carried out in order to determine the optimum concentration of encapsulation matrix.

Table 4.16: Effects of different concentrations of Sodium alginate and Calcium chloride. Observations were taken after 30 minutes of complexation process in Calcium chloride dehydrate.

$CaCl_2$ (μM)	Sodium alginate concentration			
	2%	3%	4%	5%
25	+	++	++	+++
50	+	++	+++	+++
75	++	+++	+++	++++
100	++	+++	++++	++++

\+ Very fragile bead with no definite shape

++ Fragile bead with no definite shape

+++ Soft, solid, and uniform shape

++++ Optimum, firm, uniform and round shape

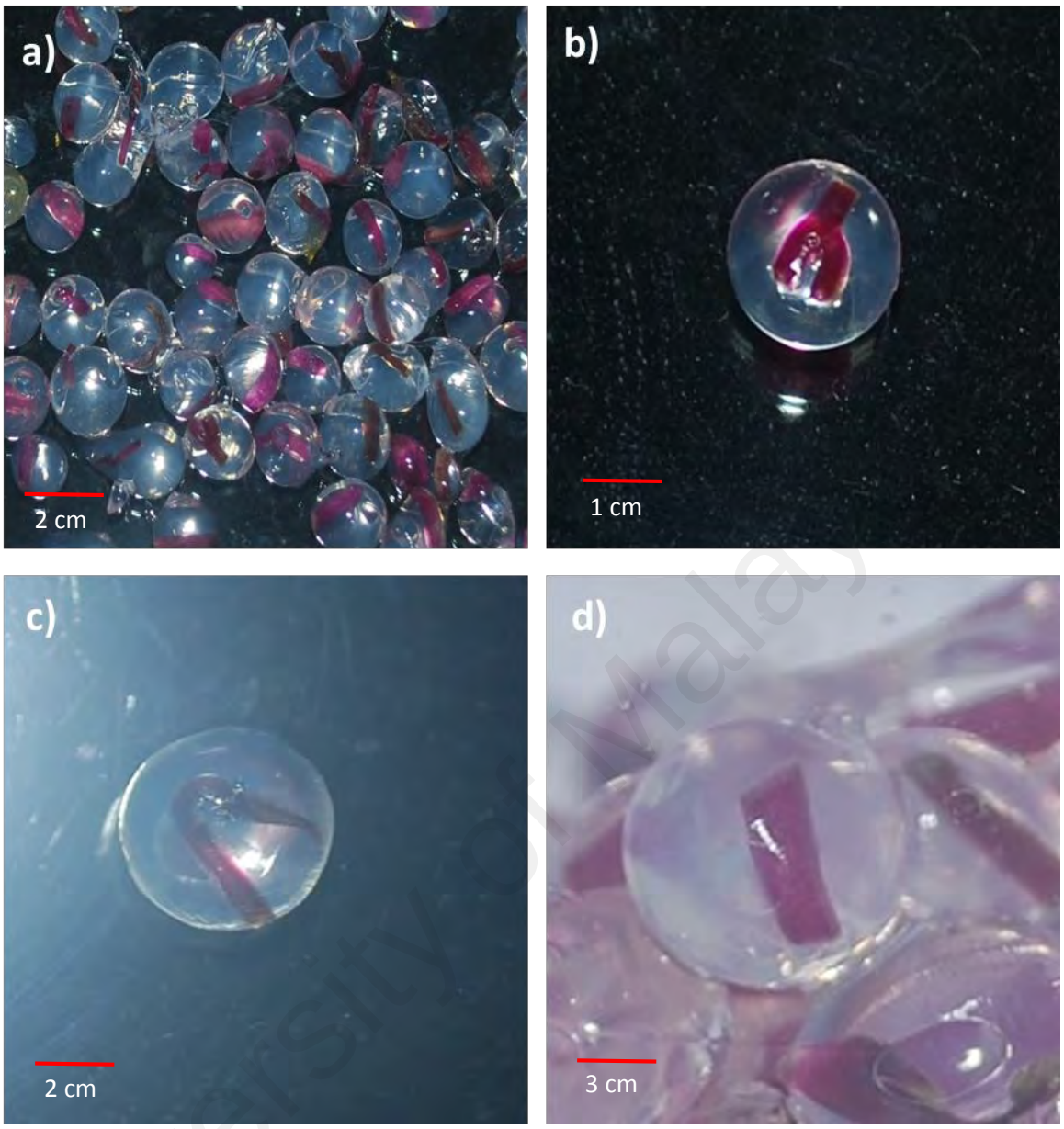


Figure 4.19: Formation of synthetic seeds. a) propagules encapsulated with Sodium alginate and harden in Calcium chloride dehydrate to form ideal beads, b) encapsulated node explant, c) encapsulated shoot tip and d) encapsulated stem explant.

4.4.2 Synthetic seed Germination on MS media

The ability of encapsulated explants to break the encapsulated matrix (bead) and germinated is also one of the criteria to determine the optimum concentration of encapsulation matrix. To evaluate the optimum concentration of encapsulation matrix, explants encapsulated with 16 different solutions of sodium alginate and calcium chloride dehydrate included (+++) were germinated on MS solid and liquid media (Figure 4.20). To perceive the germination of synthetic seeds, the capability of explant to break the bead and to continue normal growth to the emergence of shoot or/and root were observed. Figure 4.21 shows the development of synthetic seed to form complete plantlet.

Based on results presented in Tables 4.17, 4.18 and 4.19, it was obvious that the concentration of sodium alginate solution and calcium chloride solution influenced the frequency of synthetic seeds germination of *Dioscorea alata*. The encapsulated explants did not germinate at the same time. Encapsulated shoot tips germinated earlier compared to encapsulated nodes and stems. Cracking of beads was first observed with outgrowth of shoot from shoot tips explant after 5 days in culture followed with node propagules after 7 days. Encapsulated stem started to germinate after 11 days in culture without shoot or root emergence. Formation of purplish white callus was detected.

Based on the results obtained in Table 4.17, the encapsulated shoot tips germinated at different time depending on concentrations of sodium alginate and calcium chloride dehydrate used. Emergence of shoots was observed as early as 5 days from shoot tips encapsulated with 2 % sodium alginate and 25, 50, 75 and 100 mM calcium chloride dehydrate, 3 % sodium alginate and 25, 50 mM Calcium chloride solution and 4 % sodium alginate and 25 mM calcium chloride solution. Shoot tips encapsulated with 5 % sodium alginate solution and hardened in 75 and 100 mM calcium chloride dehydrate cultured on

MS solid media took the longest time to germinate which was 14 days. Shoot tips encapsulated with 3% sodium alginate solution and hardened in 75 and 100 mM calcium chloride dehydrate exhibited the highest germination percentage, (90.00 ± 0.06 %). The lowest percentage of germination, (43.33 ± 0.09) was observed from shoot tips encapsulated with 5% sodium alginate solution and hardened in 100 mM calcium chloride dehydrate.

Table 4.18 shows effect of different concentrations of Sodium alginate and calcium chloride on germination of synthetic seeds from node segments on MS solid and in liquid media. Early germination was observed from synthetic seeds cultured in MS liquid media in all concentrations of sodium alginate solution tested. Encapsulated nodes started to exhibit shoots 7 days after being cultured in liquid media and 8 days after being cultured on solid media. Node explants encapsulated with 3% sodium alginate solution and hardened in 75 and 100 mM calcium chloride dehydrate showed the highest germination percentage, 93.33 ± 0.05. The lowest percentage of germination (46.67 ± 0.09 %) was observed from node segments encapsulated with 5% sodium alginate solution hardened in 100 mM calcium chloride dehydrate.

Table 4.19 shows the effects of different concentrations of Sodium alginate and calcium chloride on germination of synthetic seeds from stem segments on MS solid and in liquid media. Stem explants encapsulated with 3% sodium alginate solution and hardened in 100 mM calcium chloride dehydrate exhibited the highest germination percentage, i.e 46.67 ± 0.09 %. The lowest percentage of germination, 13.33 ± 0.06 % was observed from stem explants encapsulated with 5% sodium alginate solution and hardened in 100 mM calcium chloride dehydrate.

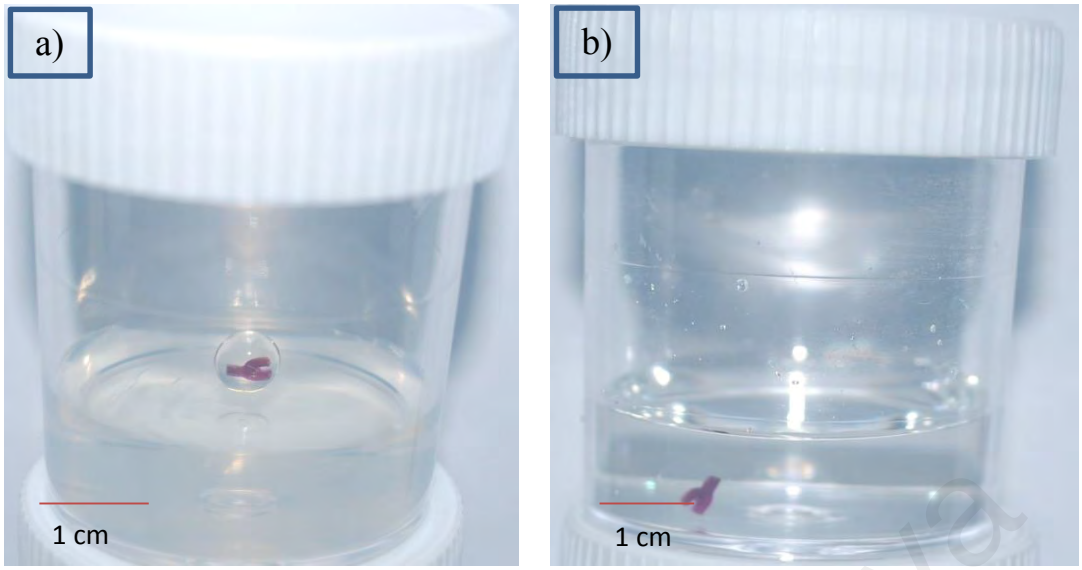


Figure 4.20: Synthetic seeds from encapsulated node explants cultured on a) MS basal solid media and b) MS basal liquid media.

Table 4.17: Effects of different concentrations of Sodium alginate and Calcium chloride on germination of synthetic seeds from shoot tips on MS solid and in liquid media maintained at 25±1^{0}C with 16 h light and 8 h dark. Thirty replicates were used in each treatment.

Sodium Alginate + Calcium choloride solution	Period of Germination (Day)		Germination Rate (%)	
	MS Solid Media	MS Liquid Media	MS Solid Media	MS Liquid Media
2% + 25 mM	5.74 ± 0.11^{e}	4.74 ± 0.11^{e}	76.67 ± 0.08^{abc}	76.67 ± 0.08^{abc}
2% + 50 mM	5.75 ± 0.15^{e}	4.75 ± 0.15^{e}	80.00 ± 0.07^{abc}	80.00 ± 0.07^{abc}
2% + 75 mM	5.75 ± 0.16^{e}	4.75 ± 0.16^{e}	80.00 ± 0.07^{abc}	80.00 ± 0.07^{abc}
2% + 100 mM	5.60 ± 0.31^{e}	4.88 ± 0.12^{e}	80.00 ± 0.07^{abc}	80.00 ± 0.07^{abc}
3% + 25 mM	6.00 ± 0.00^{e}	4.80 ± 0.15^{e}	83.33 ± 0.07^{bc}	83.33 ± 0.07^{bc}
3% + 50mM	5.92 ± 0.06^{e}	4.80± 0.13^{e}	83.33 ± 0.07^{bc}	83.33 ± 0.07^{bc}
3% + 75 mM	7.89 ± 0.09^{d}	6.89 ± 0.15^{d}	90.00 ± 0.06^{a}	90.00 ± 0.06^{a}
3% + 100 mM	7.89 ± 0.15^{d}	6.89 ± 0.13^{d}	90.00 ± 0.06^{a}	90.00 ± 0.06^{a}
4% + 25 mM	5.76 ± 0.14^{e}	4.72 ± 0.12^{e}	83.33 ± 0.07^{bc}	83.33 ± 0.07^{bc}
4% + 50 mM	7.70 ± 0.10^{d}	6.70 ± 0.10^{d}	76.67 ± 0.08^{abc}	76.67 ± 0.08^{abc}
4% + 75 mM	7.91 ± 0.06^{d}	6.64 ± 0.14^{d}	73.33 ± 0.08^{abcd}	73.33 ± 0.08^{abcd}
4% + 100 mM	8.52 ± 0.13^{c}	7.57 ± 0.13^{c}	70.00 ± 0.09^{abcd}	70.00 ± 0.09^{abcd}
5% + 25 mM	7.84 ± 0.09^{d}	6.84 ± 0.09^{d}	63.33 ± 0.09^{bcde}	63.33 ± 0.09^{bcde}
5% + 50mM	10.00 ± 0.12^{b}	8.71 ± 0.11^{b}	56.67 ± 0.09^{cde}	56.67 ± 0.09^{cde}
5% + 75 mM	14.00 ± 0.13^{a}	11.77 ± 0.12^{a}	50.00 ± 0.09^{cd}	50.00 ± 0.09^{cd}
5% + 100 mM	14.00 ± 0.11^{a}	12.00 ± 0.14^{a}	43.33 ± 0.09^{e}	43.33 ± 0.09^{e}

Means followed by the same letter are not significantly different at the 0.05 level of confidence by one way ANOVA.

Table 4.18: Effects of different concentrations of Sodium alginate and Calcium chloride on germination of synthetic seeds from node segments on MS solid and in liquid media, maintained at 25±1^{0}C with 16 h light and 8 h dark. Thirty replicates were used in each treatment.

Sodium Alginate + Calcium choloride solution	Period of Germination (Day)		Germination Rate (%)	
	MS Solid Media	MS Liquid Media	MS Solid Media	MS Liquid Media
2% + 25 mM	7.80 ± 0.15^{e}	6.80 ± 0.08^{f}	80.00 ± 0.07^{abc}	80.00 ± 0.07^{abc}
2% + 50 mM	7.83 ± 0.08^{e}	7.00 ± 0.09^{f}	80.00 ± 0.07^{abc}	80.00 ± 0.07^{abc}
2% + 75 mM	8.04 ± 0.13^{e}	7.08 ± 0.11^{f}	80.00 ± 0.07^{abc}	80.00 ± 0.07^{abc}
2% + 100 mM	8.04 ± 0.65^{e}	7.04 ± 0.16^{f}	83.33 ± 0.07^{bc}	83.33 ± 0.07^{bc}
3% + 25 mM	7.70 ± 0.09^{e}	7.04 ± 0.13^{f}	86.67 ± 0.06^{a}	86.67 ± 0.06^{a}
3% + 50mM	7.96 ± 0.14^{e}	6.89 ± 0.06^{f}	90.00 ± 0.06^{a}	90.00 ± 0.06^{a}
3% + 75 mM	9.92 ± 0.15^{d}	8.93 ± 0.15^{e}	93.33 ± 0.05^{a}	93.33 ± 0.05^{a}
3% + 100 mM	9.79 ± 0.12^{d}	9.82 ± 0.10^{d}	93.33 ± 0.05^{a}	93.33 ± 0.05^{a}
4% + 25 mM	7.93 ± 0.10^{e}	6.92 ± 0.14^{f}	90.00 ± 0.06^{a}	90.00 ± 0.06^{a}
4% + 50 mM	10.08 ± 0.05^{d}	8.85 ± 0.15^{e}	80.00 ± 0.07^{abc}	80.00 ± 0.07^{abc}
4% + 75 mM	10.00 ± 0.13^{d}	8.73 ± 0.11^{e}	76.67 ± 0.08^{abc}	76.67 ± 0.08^{abc}
4% + 100 mM	11.44 ± 0.15^{c}	11.00 ± 0.12^{b}	60.00 ± 0.09^{bcd}	60.00 ± 0.09^{bcd}
5% + 25 mM	12.06 ± 0.10^{b}	10.11 ± 0.08^{cd}	56.67 ± 0.09^{cd}	56.67 ± 0.09^{cd}
5% + 50mM	12.13 ± 0.15^{b}	10.19 ± 0.10^{c}	53.33 ± 0.09^{d}	53.33 ± 0.09^{d}
5% + 75 mM	15.93 ± 0.15^{a}	12.71 ± 0.13^{a}	50.00 ± 0.09^{d}	50.00 ± 0.09^{d}
5% + 100 mM	16.07 ± 0.16^{a}	13.00 ± 0.10^{a}	46.67 ± 0.09^{d}	46.67 ± 0.09^{d}

Means followed by the same letter are not significantly different at the 0.05 level of confidence by one way ANOVA.

Table 4.19: Effects of different concentrations of Sodium alginate and Calcium chloride on germination of synthetic seeds from stems on MS solid and in liquid media maintained at 25±1^{0}C with 16 h light and 8 h dark. Thirty replicates were used in each treatment.

Sodium Alginate + Calcium choloride solution	Period of Germination (Day)		Germination Rate (%)	
	MS Solid Media	MS Liquid Media	MS Solid Media	MS Liquid Media
2% + 25 mM	14.08 ± 0.18^{e}	11.92 ± 0.14^{d}	36.67 ± 0.09^{abc}	36.67 ± 0.09^{abc}
2% + 50 mM	14.00 ± 0.17^{e}	11.83 ± 0.11^{d}	40.00 ± 0.09^{abc}	40.00 ± 0.09^{abc}
2% + 75 mM	13.80 ± 0.29^{e}	12.20 ± 0.13^{d}	33.33 ± 0.09^{abc}	33.33 ± 0.09^{abc}
2% + 100 mM	13.89 ± 0.11^{e}	11.78 ± 0.15^{d}	30.00 ± 0.09^{abc}	30.00 ± 0.09^{abc}
3% + 25 mM	14.17± 0.11^{e}	12.00 ± 0.12^{d}	40.00 ± 0.09^{abc}	40.00 ± 0.09^{abc}
3% + 50mM	14.00 ± 0.20^{e}	12.00 ± 0.22^{d}	43.33 ± 0.09^{bc}	43.33 ± 0.09^{bc}
3% + 75 mM	15.79 ± 0.14^{d}	13.93 ± 0.13^{c}	46.67 ± 0.09^{a}	46.67 ± 0.09^{a}
3% + 100 mM	16.07 ± 0.07^{d}	14.00 ± 0.19^{c}	46.67 ± 0.09^{a}	46.67 ± 0.09^{a}
4% + 25 mM	13.81 ± 0.18^{e}	11.91 ± 0.09^{d}	36.67 ± 0.09^{abc}	36.67 ± 0.09^{abc}
4% + 50 mM	15.88 ± 0.13^{d}	14.00 ± 0.19^{c}	26.67 ± 0.08^{abc}	26.67 ± 0.08^{abc}
4% + 75 mM	15.71 ± 0.28^{d}	14.14 ± 0.14^{c}	23.33 ± 0.08^{abc}	23.33 ± 0.08^{abc}
4% + 100 mM	21.17 ± 0.17^{c}	18.83 ± 0.17^{b}	20.69 ± 0.08^{abc}	20.69 ± 0.08^{abc}
5% + 25 mM	16.14 ± 0.14^{d}	14.29 ± 0.29^{c}	22.58 ± 0.08^{abc}	22.58 ± 0.08^{abc}
5% + 50mM	16.17 ± 0.17^{d}	14.17± 0.17^{c}	20.00 ± 0.07^{abc}	20.00 ± 0.07^{abc}
5% + 75 mM	23.75 ± 0.25^{b}	20.20 ± 0.20^{a}	16.67 ± 0.07^{bc}	16.67 ± 0.07^{bc}
5% + 100 mM	24.40 ± 0.40^{a}	20.50 ± 0.50^{a}	13.33 ± 0.06^{c}	13.33 ± 0.06^{c}

Means followed by the same letter are not significantly different at the 0.05 level of confidence by one way ANOVA.

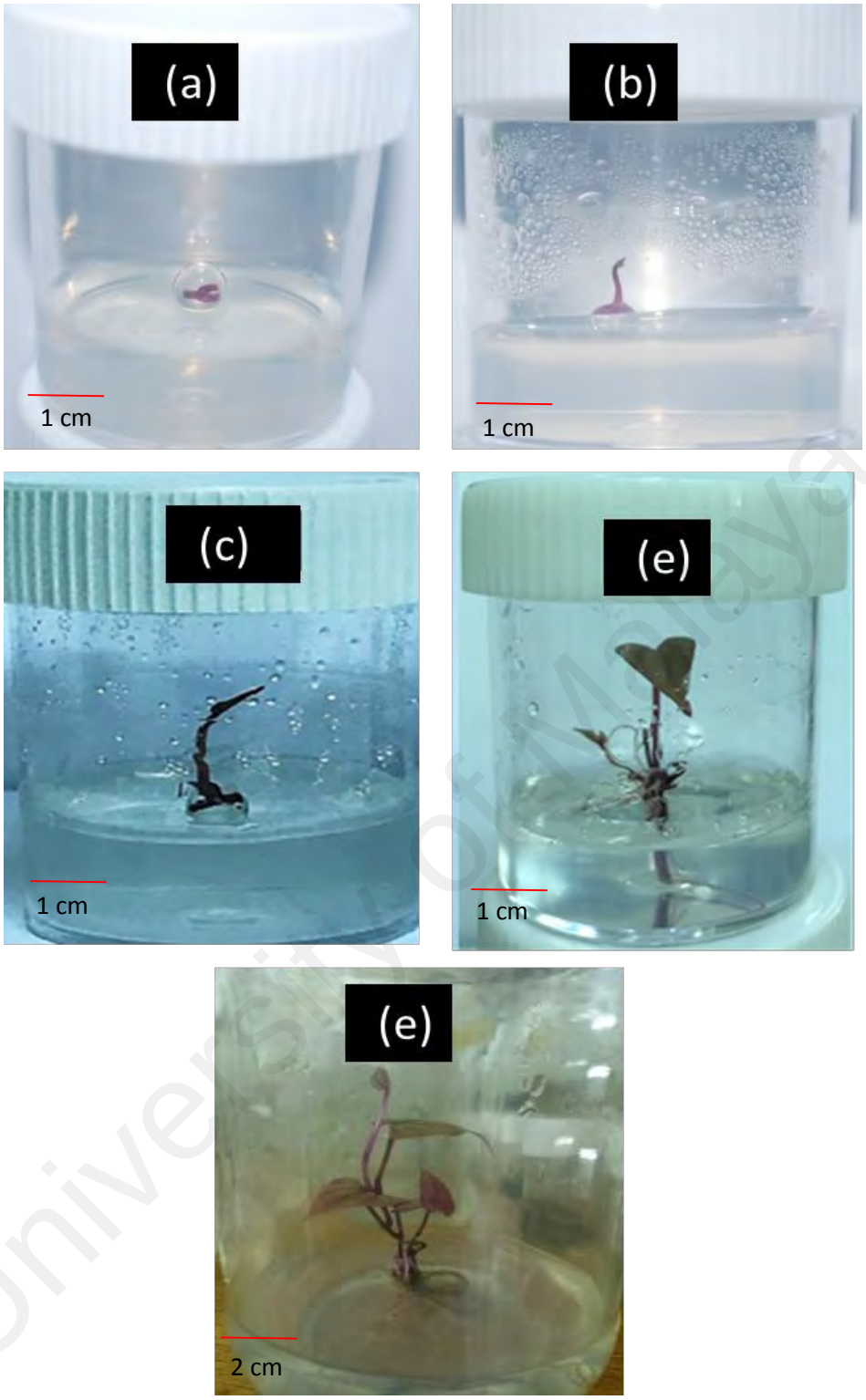


Figure 4.21: Development of synthetic seeds of *Dioscorea alata*, a) encapsulation of node explant cultured on MS basal medium, b) germination of artificial seed after 6 days in culture, c) shoot elongation after 2 weeks in culture, d) development of shoots and roots after 3 weeks and f) complete plantlet after 5 weeks in culture.

4.4.3 Effect of encapsulation matrix on Synthetic seed Germination

Synthetic seed germination and growth was affected by encapsulation matrices. Tables 4.20, 4.21 and 4.22 showed the growth responses of shoot tips, nodes and stem explants of *Dioscorea alata* in different encapsulation matrices after being transplanted onto MS media solid and liquid. Early germination was observed when shoot tips, node segments and stem explants were encapsulated with 3% sodium alginate supplemented with 1.0 mg/l BAP applied singly or in combinations with 2.0 mg/l 2,4-D. Seeds germination was observed as early as 4 days from encapsulated shoot tips cultured in MS liquid media and 5 days on MS solid media (Table 4.20). Higher germination rates were observed from propagules encapsulated with sodium alginate solution supplemented with plant growth regulators. 96.67 ± 0.03 % encapsulated shoot tips with plant growth regulators successfully germinated compared to 90.00 ± 0.06 % in control medium (encapsulated shoot tips without plant growth regulators).

Synthetic seeds from encapsulated node explants also managed to germinate earlier when encapsulated media was supplemented with 1.0 mg/l BAP or 1.0 mg/l BAP and 2.0 mg/l 2,4-D compared to node explants encapsulated with 3 % Sodium alginate only. Cracking of beads was first observed with outgrowth of microshoots after 5 days of incubation (Table 4.21). The percentage of germination also increased with the addition of plant growth regulators into the encapsulation media; 100 ± 0.00 of encapsulated node segments managed to germinate in liquid and solid MS media.

Early germination also observed from stem explants encapsulated with 3 % sodium alginate hardened in 75 or 100 mM fortified with 1.0 mg/l BAP applied singly or 1.0 mg/l BAP and 2.0 mg/l 2,4-D compared to stem explants encapsulated with 3% sodium alginate only (Table 4.22). Callus formation was observed as early as 9 days when

encapsulated stem explants was cultured in liquid media and 11 days on solid media. Germination rates of synthetic seeds also increased when plant growth regulators (BAP and 2,4-D) was added into the encapsulation media. For example, 56.67 ± 0.09 % encapsulated stem explants with 3% Sodium alginate hardened in 75 mM calcium chloride dehydrate supplemented with 1.0 mg/l BAP singly or in combinations with 2.0 mg/l 2,4-D and 53.33 ± 0.09 % encapsulated shoot tips with 3% Sodium alginate hardened in 100 mM calcium chloride dehydrate successfully germinated compared to 46.67 ± 0.09 % in control medium.

Table 4.20: Growth responses of encapsulated shoot tips of *Dioscorea alata* in different encapsulation matrices after being transplanted onto MS solid and liquid media maintained at 25±1^{0}C with 16 h light and 8 h dark. Thirty replicates were used in each treatment.

Sodium Alginate + Calcium choloride solution	Period of Germination (Day)		Germination Rate (%)	
	MS Solid Media	MS Liquid Media	MS Solid Media	MS Liquid Media
3% + 75 mM	7.89 ± 0.09^a	6.89 ± 0.15^b	90.00 ± 0.06^a	90.00 ± 0.06^a
3% + 75mM + 1.0 BAP	5.07 ± 0.11^c	4.00 ± 0.12^c	96.67 ± 0.03^a	96.67 ± 0.03^a
3% + 75 Mm + 1.0 BAP + 2.0 2,4-D	5.00 ± 0.11^c	3.97 ± 0.03 ^{c}	96.67 ± 0.03^a	96.67 ± 0.03^a
3% + 100 mM	7.89 ± 0.17^b	6.89 ± 0.13^a	90.00 ± 0.06^a	90.00 ± 0.06^a
3% + 100 Mm + 1.0 BAP	4.97 ± 0.08^c	4.14 ± 0.10^c	96.67 ± 0.03^a	96.67 ± 0.03^a
3% + 100 Mm + 1.0 BAP + 2.0 2,4-D	4.93 ± 0.07^c	3.93 ± 0.14^c	96.67 ± 0.03^a	96.67 ± 0.03^a

Means followed by the same letter are not significantly different at the 0.05 level of confidence by one way ANOVA.

Table 4.21: Growth responses of encapsulated nodes of *Dioscorea alata* in different encapsulation matrices after being transplanted onto MS solid and liquid media maintained at 25±1^{0}C with 16 h light and 8 h dark. Thirty replicates were used in each treatment.

Sodium Alginate + Calcium choloride solution	Period of Germination (Day)		Germination Rate (%)	
	MS Solid Media	MS Liquid Media	MS Solid Media	MS Liquid Media
3% + 75 mM	9.92 ± 0.15^a	8.93 ± 0.15^a	93.33 ± 0.05^a	93.33 ± 0.05^a
3% + 75mM + 1.0 BAP	5.97 ± 0.14^b	5.00 ± 0.14^b	100 ± 0.00^a	100 ± 0.00^a
3% + 75 Mm + 1.0 BAP + 2.0 2,4-D	5.87 ± 0.15^b	4.73 ± 0.08^b	100 ± 0.00^a	100 ± 0.00^a
3% + 100 mM	9.79 ± 0.12^a	9.82 ± 0.10^a	93.33 ± 0.05^a	93.33 ± 0.05^a
3% + 100 Mm + 1.0 BAP	5.86 ± 0.06^b	5.00 ± 0.08^b	100 ± 0.00^a	100 ± 0.00^a
3% + 100 Mm + 1.0 BAP + 2.0 2,4-D	5.77± 0.08^b	4.83 ± 0.07^b	100 ± 0.00^a	100 ± 0.00^a

Means followed by the same letter are not significantly different at the 0.05 level of confidence by one way ANOVA.

Table 4.22: Growth responses of encapsulated stems of *Dioscorea alata* in different encapsulation matrices after being transplanted onto MS solid and liquid media maintained at 25±1^{0}C with 16 h light and 8 h dark. Thirty replicates were used in each treatment.

Sodium Alginate + Calcium choloride solution	Period of Germination (Day)		Germination Rate (%)	
	MS Solid Media	MS Liquid Media	MS Solid Media	MS Liquid Media
3% + 75 mM	15.79 ± 0.14^b	13.93 ± 0.13^b	46.67 ± 0.09^a	46.67 ± 0.09^a
3% + 75mM + 1.0 BAP	11.00 ± 0.15^{cd}	8.94 ± 0.16^d	56.67 ± 0.09^a	56.67 ± 0.09^a
3% + 75 Mm + 1.0 BAP + 2.0 2,4-D	10.76± 0.10^d	8.76 ± 0.11^d	56.67 ± 0.09^a	56.67 ± 0.09^a
3% + 100 mM	16.07 ± 0.07^a	14.00 ± 0.19^a	46.67 ± 0.09^a	46.67 ± 0.09^a
3% + 100 Mm + 1.0 BAP	12.00 ± 0.16^b	9.87 ± 0.09^c	53.33 ± 0.09^a	53.33 ± 0.09^a
3% + 100 Mm + 1.0 BAP + 2.0 2,4-D	11.44 ± 0.20^c	9.75 ± 0.11^c	53.33 ± 0.09^a	53.33 ± 0.09^a

Means followed by the same letter are not significantly different at the 0.05 level of confidence by one way ANOVA.

4.4.4 Effect of storage period on Synthetic seed Germination

The effect of storage period on germination of synthetic seeds produced was presented in Table 4.23. The percentage of germination of synthetic seeds (encapsulated shoot tips with 3.0 % sodium alginate hardened in 100 mM calcium chloride dehydrate fortified with 1.0 mg/l BAP and 2.0 mg/l 2,4-D) cultured on MS basal media was 100.00 ± 0.00 % without storage. Cracking of beads was observed with outgrowth of shoot tips after 4 days of culture period. After 7 days of storage at 4 ± 1 ºC, the germination rate of encapsulated shoot tips was 74.19 ± 0.08 %. Cracking of beads was observed after 8 days of culture period. Germination rate dropped to 30.00 ± 0.09 % after 14 days of storage. After 21 days in storage, low germination rate 06.67 ± 0.05 % was recorded. Only 03.33 ± 0.03 % of synthetic seeds germinated after 30 days of storage. The synthetic seeds totally lost the seed viability after 40 days stored at 4 ± 1 ºC.

Table 4.23: Effects of storage period at 4 ± 1 ºC on germination of synthetic seeds of *Dioscorea alata* (shoot tips propagules encapsulated with 3% sodium alginate supplemented with 1.0 mg/l BAP and 2.0 mg/l 2,4-D) on MS basal medium maintained at 25±1^{0}C with 16 h light and 8 h dark. Thirty replicates were used in each treatment.

Storage period (Day)	No. of synthetic seeds	No. of germinated seeds	Germination rates (% ± SE)
0	30	30	100.00 ± 0.00^a
7	30	22	74.19 ± 0.08^b
14	30	9	30.00 ± 0.09^c
21	30	3	10.00 ± 0.06^d
30	30	1	03.33 ± 0.03^d
40	30	0	0.00 ± 0.00^d

Means followed by the same letter are not significantly different at the 0.05 level of confidence by one way ANOVA.

4.5 Acclimatization of Plantlets of *Dioscorea alata* L.

4.5.1 Determination of Soil content

Growth substrates play crucial role/factor in acclimatization process. In order to determine the best substrate for *Dioscorea alata* acclimatization and growth, four substrates which are Black soil, Red soil, Vermicompost and Culite were analysed to determine the amount of plant available nutrients in the soils included Exchangeable Bases (K, Ca and Mg), Cation Exchange Capacity (CEC), Nitrogen (N), Phosphorous (P), Potassium (K), Total Phosphorous, Available Phosphorous and moisture content of the substrates. Results of soil analysis was presented in Table 4.24.

Soil pH is the measure of acidity or alkalinity of the substrate tested. The pH scale goes from 0.0 to 14.0. A substrate gets more acidic as the pH values decrease from 7.0 to 0.0 and is more alkaline as pH values increase from 7.0 to 14.0. From the results obtained the pH for Black soil was slightly alkaline with pH reading 7.58, Red soil was slightly acidic with pH 6.84, Vermicompost was slightly alkaline with pH 7.41 and Culite was moderately alkaline with pH 7.88. The increase of pH value is dependent of the base saturation. The availability of the cation can be taken up by plants increases with increase in base saturation. Culite showed the highest base saturation with a value of 39.61.

Cation exchange capacity (CEC) is the capacity of the substrate to hold cations in soil particles surfaces. CEC was calculated from the analysis results of potassium (% K), calcium (% Ca), magnesium (% Mg), sodium (% Na) and Buffer pH (% H). CEC was effected by the soil clay and organic matter content, so CEC can be considered a generalized indicator of soil texture. The value of CEC was different among the four types of sowing media. Black soil and Red soil typical texture were loamy soils with CEC 15.57

meq/100 g and 15.03 meq/100 g respectively. Culite typical texture was clay soil with CEC 33.55 meq/100 g. High CEC, 88.77 meq/100 g was observed from Vermicompost.

Potassium (K) is an essential element that associate with water movement, nutrient and carbohydrate in plant tissue. Based on Table 7.1, Black soil contained high K with 0.85 meq/100g. Calcium (Ca) is one of the elements that is constituent of the cell wall and apparently influences its elasticity. Culite contained the highest Ca (12.27 meq/100g) and Black soil possessed the lowest Ca (0.32 meq/100g). Magnesium (Mg) is an important element constituent of chlorophyll and hence plays important role in photosynthesis process. Red soil was detected with the lowest Mg content (0.28 meq/100g). Sodium (Na) may indicate potential for soil structure breakdown and water percolation problem. Black soil (0.11 meq/100g), Red soil (0.06 meq/100g) and Culite (0.34 meq/100g) showed very low sodium hazard. Vermcompost with high Na (6.21 meq/100g) was low sodium hazard.

Percentage of organic carbon is the measure of the percentage of total carbon-containing materials in a substrate. The organic matter content may affect certain herbicide application rate and may affect fertilizer rate recommendation for nitrogen sulfur. Vermicompost contained the highest percentage of organic carbon (21.19 %). Red soil contained the lowest percentage of organic carbon (0.36 %). Total Nitrogen measures the total amount of N present in the soil, much of which is held in organic matter and not immediately available to plants. Black soil, Red soil and Culite contained very low total N with the reading of 0.04, 0.03 and 0.03, respectively. Vermicompost showed high total N with the reading of 0.34.

Soil contained two types of phosporous which were Available P and Total P. Available P is the readily available phosphorous and Total P is some component of the phosphorous which becomes available or may be available when available P was depleted by plant uptake. From the results, Black soil showed extremely low Available P (7.0) but high Total P (388). Red soil contained moderate Available P (150.0) and low Total P (107.0). Extremely low Available P (4.0) and moderate Total P (209.0) was detected in Vermicompost. In culite, the Available P was moderate (197.0) while the Total P was high (391.0).

Plants also need small quantities of micronutrients such as iron (Fe), manganese (Mn), copper (Cu) and zinc (Zn), known as trace elements. Although only required in small amounts, trace elements are essential for plant growth. Critical levels for trace elements will vary between soil types and plants. From the results in Table 7.1, Culite contained the highest Fe (230.6 ppm). The second highest Fe, (151.6 ppm) was observed in Red soil. Black soil and Vermicompost contained high Mn with total ppm 49.5 and 46.1, respectively. The highest Cu (0.83 ppm) was detected in Vermicompost. The highest trace element Zn was observed in Vermicompost with total ppm of 7.21.

Table 4.24: Nutrient content of different sowing media (Black soil, Red soil, Vermicompost and Culite).

Nutrient level	Sowing Media			
	Black Soil	**Red Soil**	**Vermi-compost**	**Culite**
Soil pH	7.58	6.84	7.41	7.88
Organic Carbon (%)	2.74	0.36	21.19	0.57
Total Nitrogen (%)	0.04	0.03	0.34	0.03
C/N Ratio	68.00	11.00	62.00	17.00
Total P	338.00	107.00	209.00	391.00
Available P	7.00	150.00	4.00	197.00
Exchangeable cation (meq/100 g)				
Potassium	0.85	0.39	1.91	0.20
Calcium	0.32	3.23	7.20	12.27
Magnesium	0.63	0.28	0.52	0.48
Natrium	0.11	0.06	6.21	0.34
CEC (meq/ 100 g)	15.57	15.03	88.77	33.55
Bases Saturation	12.27	26.34	17.84	39.61
Trace Elements (Total ppm)				
Fe	60.90	151.60	23.30	330.60
Mn	49.50	11.10	41.60	13.60
Cu	0.35	0.34	0.83	0.20
Zn	5.42	1.35	2.21	0.95

4.5.2 Survival rate of *Dioscorea alata* L. Micropropagated plantlets

Complete micropropagated plantlets with roots (Figure 4.22) were successfully transferred to *ex vitro* environment. A great care was taken to minimize exposure to extreme light intensity. High humidity was maintained by covering potted plantlets with plastic covers (Figure 4.23) and the cover was removed in stages, creating partial exposure of the plantlets to the sunlight and full exposure after 30 days in the greenhouse (Figure 4.24). By this treatment, the plantlets were able to acclimatize progressively and grow vigorously (Table 4.25). The data recorded on the survival rate of the plantlets during acclimatization was presented in Table 4.25.

The results showed that plantlets responded positively when acclimatized in all sowing media tested; Black soil, Red soil and combination of Black soil and Red soil in the ratio of 2:1). Plantlets acclimatized with the mixture of Black soil and Red soil gave the highest survival rate for acclimatization with the percentage of 93.33 ± 0.05 % followed by Black soil with percentage of 83.33 ± 0.09 %. The lowest survival rate (70.00 ± 0.09 %) was observed from plantlets acclimatized in Red soil. However, the percentage of survival rates were declined in all treatments after 8 weeks being acclimatized. In the mixture of Black soil and Red soil, a decline of 3.0 % was observed with the percentage of 90.00 ± 0.06 %. Black soil and Red soil showed the survival rates of 73.33 ± 0.08 % and 56.67 ± 0.09 %, respectively.

Table 4.25: Responses showed by *in vitro Dioscorea alata* plantlets after being acclimatized in various sowing media. Results obtained after 4 and 8 weeks being acclimatized.

Acclimatized plantlets on different sowing substrates	Observations	Survival rates (%)
4 weeks		
Black soil.	Plantlets survived and showed healthy growth.	83.33 ± 0.09^{ab}
Red soil	Plantlets survived and showed healthy growth.	70.00 ± 0.09^{b}
Black soil + Red soil	Plantlets survived with slow growth	93.33 ± 0.05^{a}
8 weeks		
Black soil.	Plantlets survived and showed healthy growth.	73.33 ± 0.08^{ab}
Red soil	Plantlets survived and showed healthy growth.	56.67 ± 0.09^{b}
Black soil + Red soil	Plantlets survived with slow growth	90.00 ± 0.06^{a}

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

4.5.3 Macromorphological Studies of *in vivo*, *in vitro* and Acclimatized plantlets of *Dioscorea alata*.

When transferred onto different substrates, the plantlets developed from cutting nodal segments gave rise to healthy plants after 4 weeks and continue to grow vigorously after 8 weeks being acclimatized. No apparent morphological variation was observed between the *in vivo*, *in vitro* growth and acclimatized plantlets. The observation on the macromorphological of the plantlets during acclimatization was presented in Table 4.26. The plant grew well and attained 8-10 cm in height within 4 weeks of transfer (Figure 4.24). The stem was herbaceous vine with internodes square in cross section, cylindrical and purple in colour. The same characteristic of stem was observed from intact or *in vivo* plant (Table 4.1). Ovule, narrowly heart shape and green with slightly purple in colour leaves was observed. Each plant had an average number of leaves of 9.50 ± 0.92 and average number of nodes of 9.50 ± 0.92.

After 8 weeks being acclimatized, the plant height increased with mean height of 15.00 ± 0.15 cm and the stem colour was purple. Stems started to twine around the stand prepared (Figure 4.25). The ovule was maintained with narrowly heart shape and colour, the size of leaves became bigger and the number of leaves also increased with average no of 15.7 ± 0.09. Number of nodes also increased to 15.7 ± 0.09. From the observation, average number of nodes was the same as average number of leaves produced by plantlet.

Table 4.26: Responses observed in *in vitro Dioscorea alata* plantlets after being acclimatized in various sowing media. Results obtained after 1 month being acclimatized.

Parameters	***In vitro***	**4 weeks Acclimatized plantlets**	**8 weeks Acclimatized plantlets**
Stem			
Height (cm)	5.50±0.09[c]	9.00±0.15[b]	15.00±0.15[a]
Shape	Cylindrical, herbaceous vine with internodes square in cross section.	Cylindrical, herbaceous vine with internodes square in cross section.	Cylindrical, twining herbaceous vine with internodes square in cross section.
Colour	Purple colour.	Purple colour.	Purple colour.
Leaves			
No. of leaves	4.53±0.93[c]	9.50±0.92[b]	15.7±0.09[a]
Shape	Ovule, narrowly heart shape leaves.	Ovule, narrowly heart shape leaves.	Ovule, narrowly heart shape leaves.
Colour	Purple	Green with slightly purple in colour.	Green with slightly purple in colour.
No. of node	4.53±0.93[c]	9.50±0.92[b]	15.7±0.09[a]

Mean values with different letters within a column are significantly different at p=0.05, by one way ANOVA and Duncan's Multiple Range Test (DMRT).

Figure 4.22: Four-months-old well rooted complete plantlet of *Dioscorea alata* L. obtained from MS medium supplemented with 1.0 mg/l BAP and 0.5 mg/l NAA.

Figure 4.23: Plastic covering the Four-week-old *Dioscorea alata* L. plantlet after being transferred to the growth media (combinations of Black soil and Red soil).

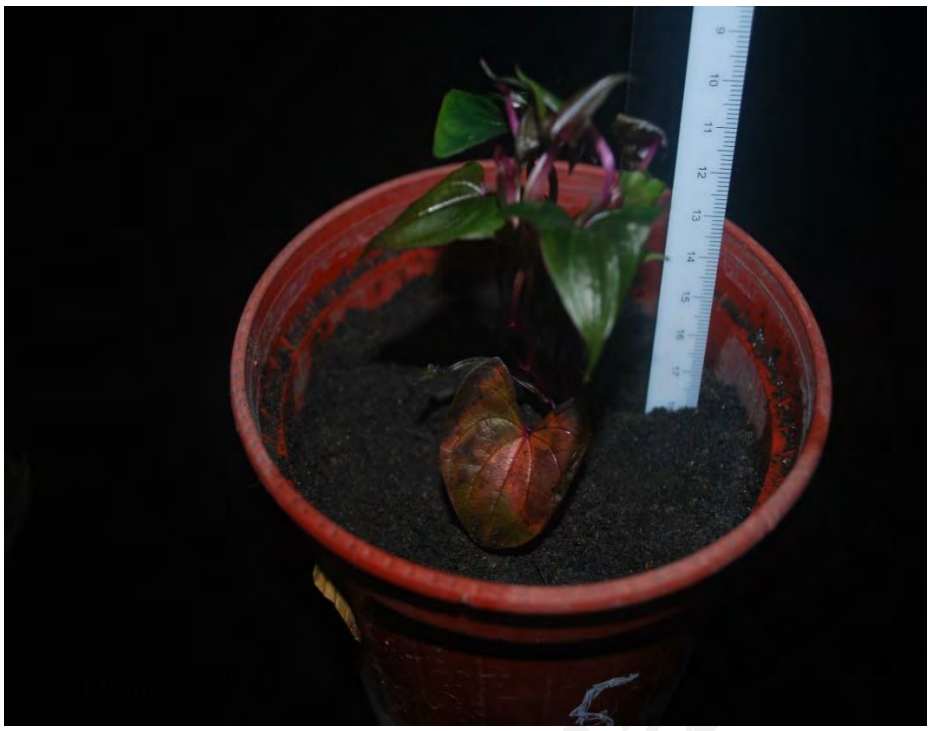


Figure 4.24: Four-week-old *Dioscorea alata* L. plantlet after being transferred to the growth media (combinations of Black soil and Red soil), for adaptation process to the natural environment with plastic cover was removed.

Figure 4.25: 8-week-old *Dioscorea alata* L. plantlet growing vigorously after being transferred to the growth media (combinations of Black soil and Red soil) under natural environment.

4.6 Anthocyanin Content & Accumulation in *Dioscorea alata* L.

4.6.1 Spectral Characteristic of Crude Anthocyanin Extract of *Dioscorea alata*

The total anthocyanin content was estimated according to the spectrophotometric pH differential method (Gabriela et al., 2010; Lee et al., 2005). Samples were diluted separately with potassium chloride buffer (pH 1) and sodium acetate buffer (pH 4.5). Increasing of pH (1.0 to 4.5) affects the concentration of the anthocyanin as well as the colour. At pH 1.0, the extracts colour become red and at pH 4.5 the colour faded and become lighter (Figure 4.26) due to the change of pigment's structure. The total concentration of anthocyanin content was also affected by the pH in the buffer as shown in Figure 4.27.

Figured 4.27 showed spectral characteristic of crude anthocyanin extract of *Dioscorea alata* callus in pH 1.0 and pH 4.5 buffers. Spectrophotometric measurement carried out clearly showed that the wavelength of maximum absorption for anthocyanin of *Dioscorea alata* was 530 nm with absorbance value of 0.3802 in pH 1.0 potassium chloride buffer. Unlike pH 4.5, the curvature of pH 1.0 was more obvious in the visible region between 460 to 560 nm. There was almost no peak of absorbance for pH 4.5 as the absorption band increased very slightly between 530 to 550 nm. The measurements were performed within 3 min after the sample was diluted with buffers to prevent misleading measurement as it was found that absorbance increase with time after dilution.

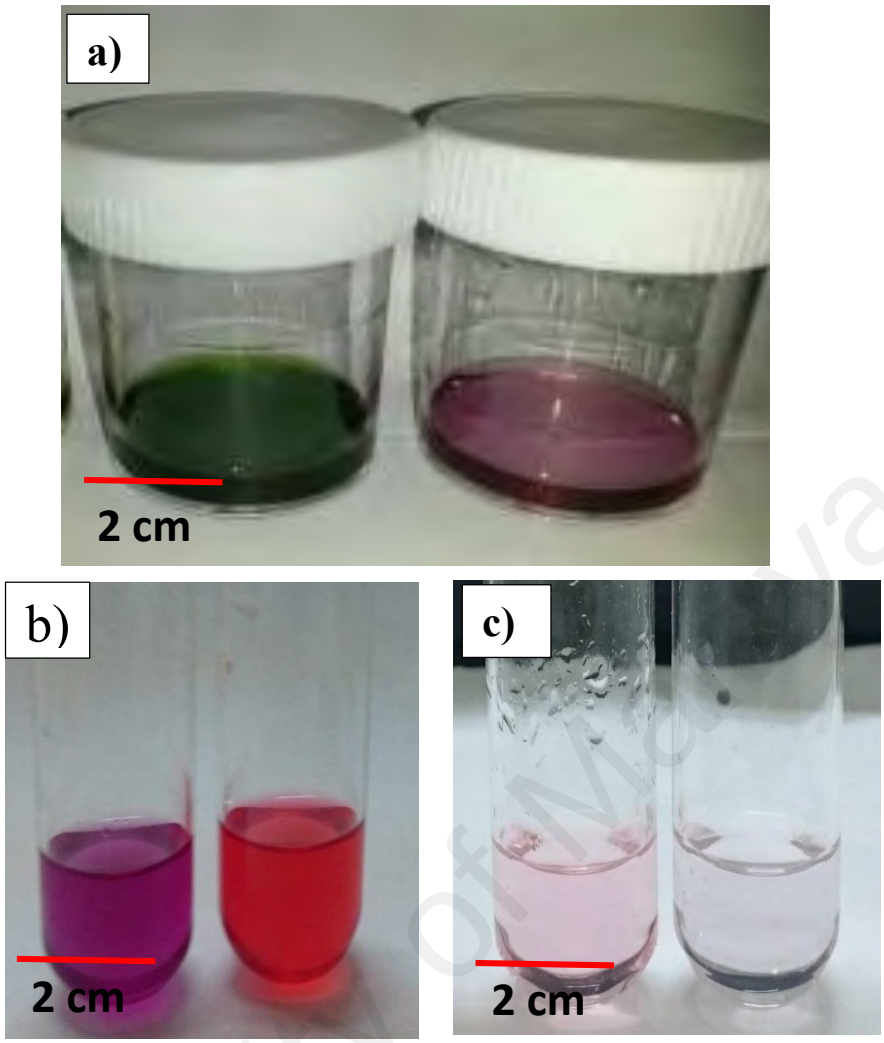


Figure 4.26: a) Crude anthocyanin extract from leaves (green) and tuber (purple) of *Dioscorea alata*, b) Colour changing in pH 1.0 buffers c) Colour changing in pH 4.5 buffers.

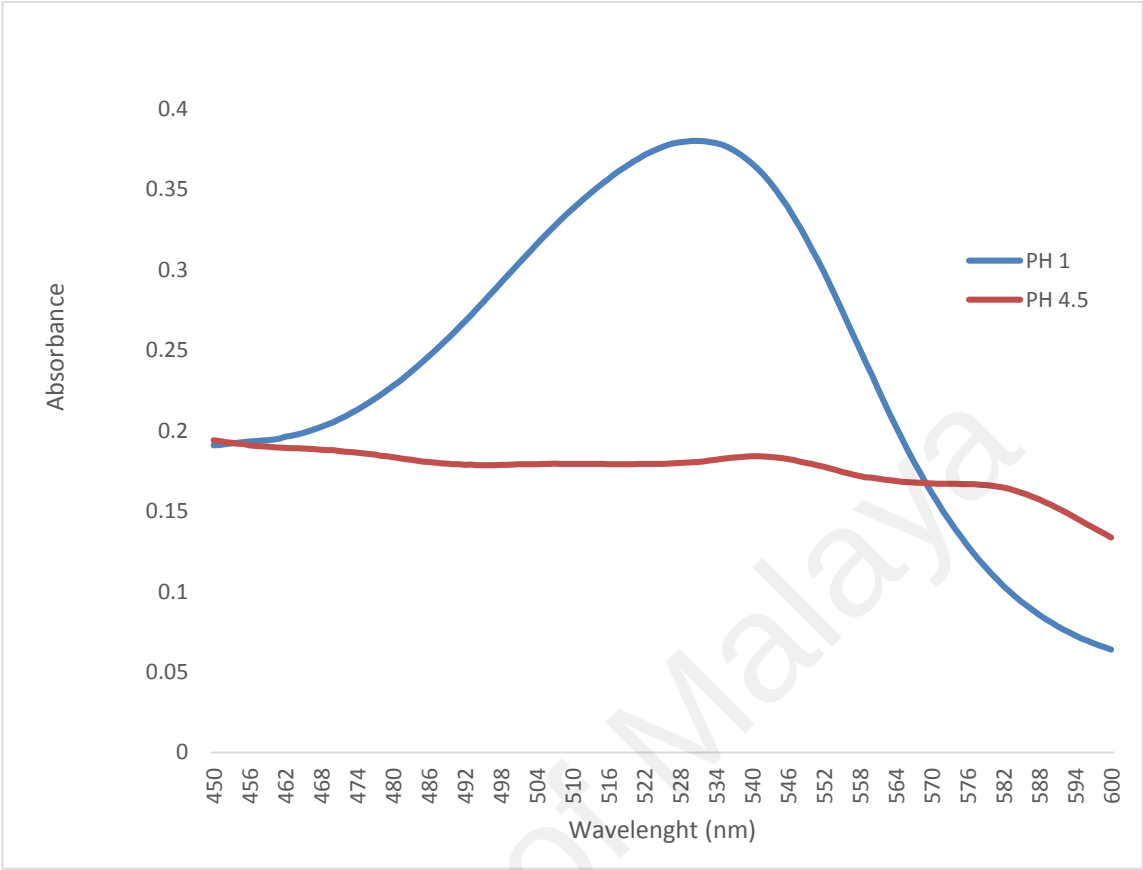


Figure 4.27: Spectral characteristic of crude anthocyanin extract of *Dioscorea alata* tuber in pH 1.0 and pH 4.5 buffers.

4.6.2 Anthocyanin content in *in vivo* and *in vitro* samples of *Dioscorea alata*

Based on several investigations, compounds produced in an *in vivo* plant could be produced at the same or different level or not produced at all in an *in vitro* grown plant. Table 4.27 showed anthocyanin production in *in vivo* and *in vitro* samples of *Dioscorea alata*. Based on Figure 4.27, 530 nm was the ($\lambda_{vis-max}$) used to calculate total monomeric anthocyanin. By using pH differential methods for anthocyanin quantification, higher anthocyanin content was observed from *in vivo* samples. The highest anthocyanin content, 341.99 ± 0.18 mg cya-3-glu / 100 g fresh weight (FW) was observed in *in vivo* tuber of *Dioscorea alata*. In leaves sample, 247.92 ± 0.71 mg cya-3-glu / 100 g FW anthocyanin content was detected. However, significant content of anthocyanin was detected in *in vitro* callus sample, 295.21 ± 0.20 mg cya-3-glu / 100 g FW and plantlet, 175.25 ± 0.42mg cya-3-glu / 100 g FW.

Table 4.27: Total monomeric anthocyanin content in *in vivo* and *in vitro* samples of *Dioscorea alata* L.

Sample	Anthocyanin content (mg cya-3-glu / 100 g FW) ± SE
In vivo (tuber)	341.99 ± 0.18*
In vivo (leaves)	247.92 ± 0.71*
In vitro (callus)	295.21 ± 0.20*
In vitro (leaves)	175.25 ± 0.42*

* Significant difference at p=0.05 (Independent Samples T-test), n=3

4.6.3 Effect of Light on Anthocyanin Accumulation in Callus of *Dioscorea alata*

Light plays crucial role in anthocyanin synthesis. Same as in *in vivo* condition, anthocyanin synthesis in *in vitro* condition also influence by light. Different colours of callus were produced at different photoperiod (Section 4.2). Four different colours of callus were observed from explants cultured on MS medium supplemented with 1.0 mg/l BAP and 2.0 mg/l 2,4-D added with 0.5 mg/l myo-inositol about 2 weeks in incubation maintained at 24L/0D, 16L/8D, 12L/12D and 0 L/24 D, respectively (Figure 4.10). After 8 weeks in culture the pigment become more visible and anthocyanin accumulation were comparable among the treatment.

The total anthocyanin content was calculated according to the spectrophotometric pH differential method by Gabriela et al. (2010) and Lee et al. (2005). Figure 4.28 showed the changing of extract colours when diluted in pH 1 and pH 4.5. At pH 1, the purple colour of callus extract change to red and almost no colour was observed at pH 4.5. Figure 4.29, shows spectral characteristic of crude anthocyanin extract of *Dioscorea alata* callus exposed under 4 different photoperiod in pH 1.0 and pH 4.5 buffers. Spectrophotometric measurement carried out for the 4 different coloured callus clearly showed that the wavelength of maximum absorption for anthocyanin was 530 nm in pH 1.0 potassium chloride buffer. The curvature of pH 1.0 was noticeable in the visible region between 460 to 560 nm. There was almost no peak of absorbance for pH 4.5 as the absorption band increased very slightly between 530 to 550 nm. Based on this spectral characteristic in Figure 8.4, total monomeric anthocyanin for callus exposed to different light treatments were estimated with 530 nm was the ($\lambda_{vis-max}$).

Table 4.28 showed total monomeric anthocyanin content in different coloured callus exposed to different photoperiod. Extract solution from coloured callus maintained in

16L/8D with red purplish colour showed the highest anthocyanin content (295.21 ± 0.20 mg cya-3-glu / 100 g FW) compared to other treatments. Higher content of anthocyanin also detected in callus maintained in 24 hours light without dark treatment (24L/0D). The purplish dark callus contained 245.46 ± 0.13 mg cya-3-glu / 100 g FW total anthocyanin. Coloured callus (purplish white) maintained in darkness for 24 hours without light exposure, (0L/24D) exhibited the lowest total monomeric anthocyanin content, (90.35 ± 0.32 mg cya-3-glu / 100 g FW).

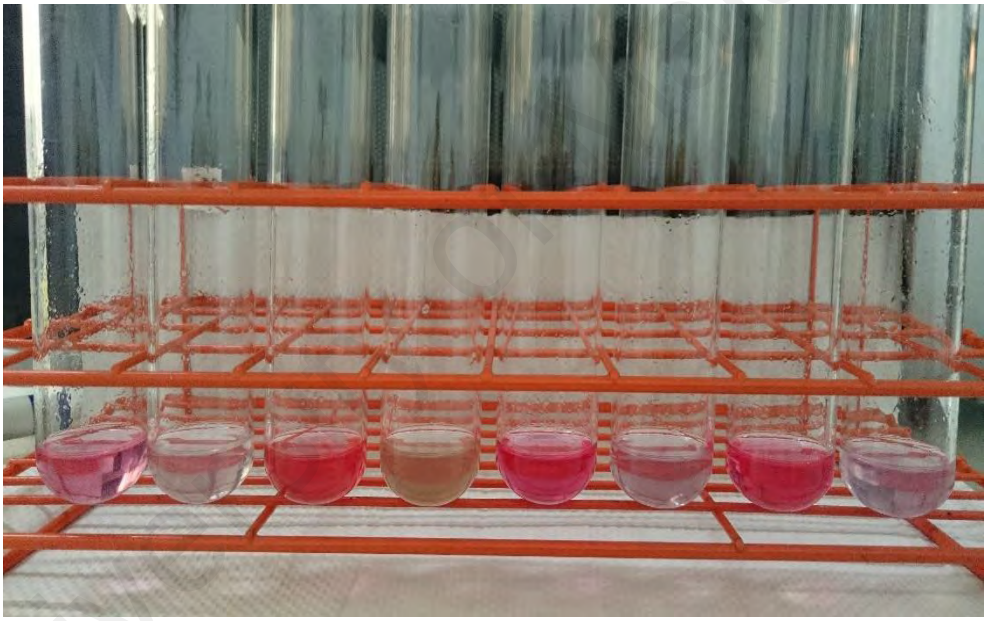


Figure 4.28: Crude anthocyanin extract from callus exposed at different photoperiod in pH 1.0 and pH 4.5 buffers.

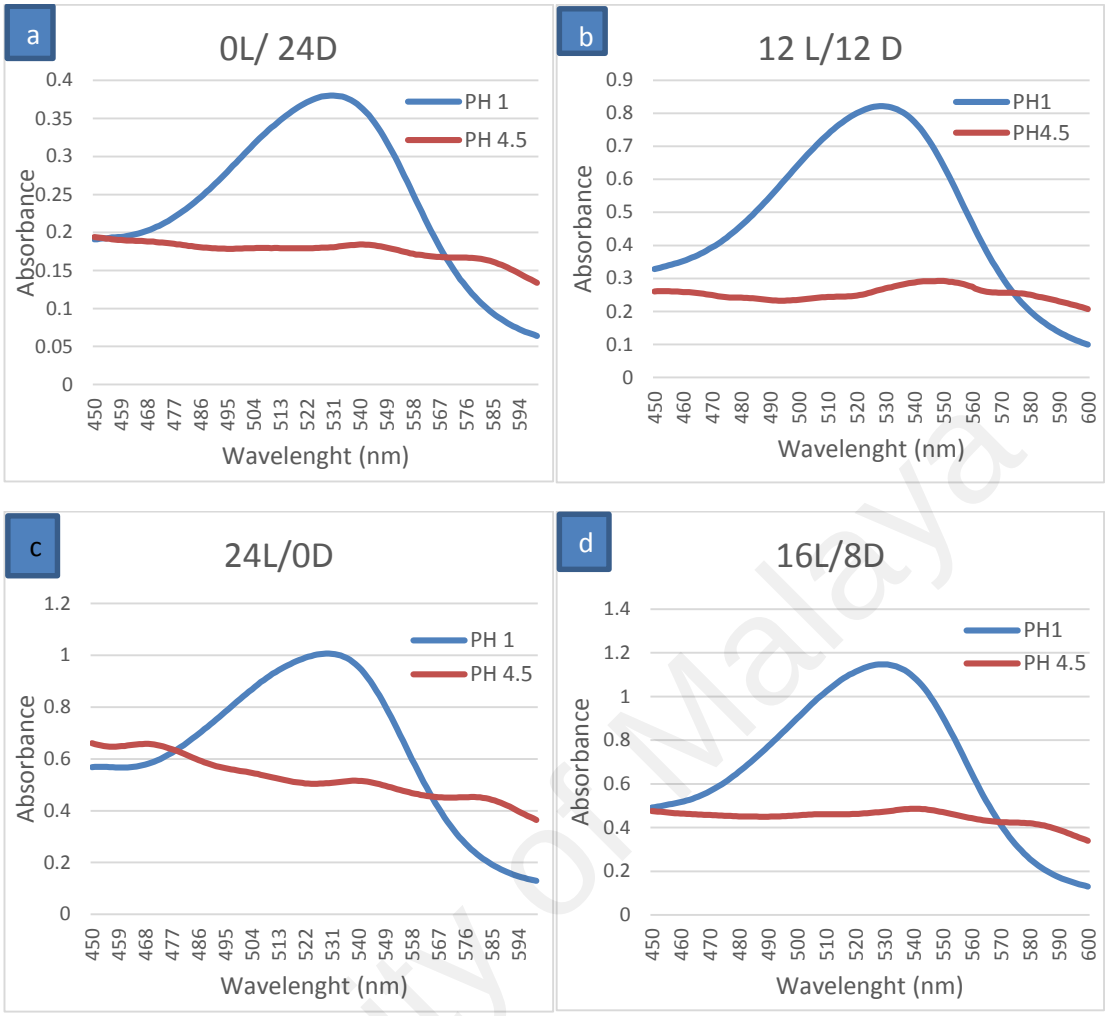
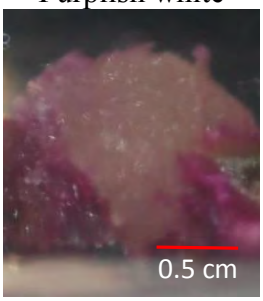
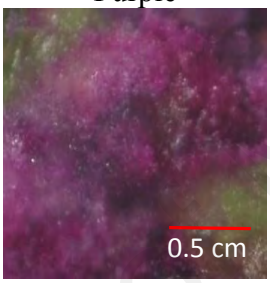
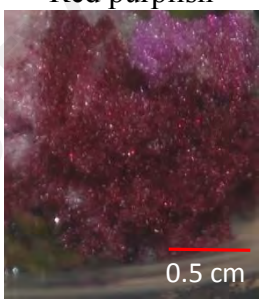
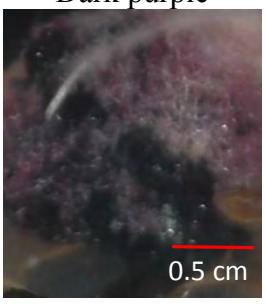


Figure 4.29: Spectral characteristic of crude anthocyanin extract of *Dioscorea alata* callus subjected to different photoperiod a) 0 Light/ 24 Dark, b) 12 Light/ 12 Dark, c) 16 Light/ 8 Dark and d) 24 Light/ 0 Dark in pH 1.0 and pH 4.5 buffers.

Table 4.28: Effect of photoperiod on caloured callus formation and total monomeric anthocyanin content in caloured callus of *Dioscorea alata* L.

Photoperiod	Caloured callus formation after 8weeks exposed to different photoperiod	Anthocyanin content (mg cya-3-glu / 100 g FW) ± SE
0 Light/ 24 Dark	Purplish white 	90.35 ± 0.32*
12 Light/ 12 Dark	Purple 	221.36 ± 0.08*
16 Light/ 8 Dark	Red purplish 	295.21 ± 0.20*
24 Light/ 0 Dark	Dark purple 	245.46 ± 0.13*

* The mean difference is significant at the 0.05 level (Independent Samples T-test), n=3

4.6.4 Phenolic acids and flavonoids content of *Dioscorea alata* L.

Phenolic acids and flavonoids are two major polyphenolic compounds that can be attributed to the anthocyanin capacity in plants. Table 4.29 exhibits the total content of phenolic and flavonoids compound in *vivo* and *in vitro* samples of *Dioscorea alata*. The results showed that both samples contained both phenolic and flavonoids compounds. The total phenolic content of *in vivo* sample was 73 mg 100 g^{-1} and *in vitro* sample was 52 mg 100 g^{-1}. While total flavonoids of *in vivo* sample was 48 mg 100 g^{-1} and *in vitro* sample was 21mg 100 g^{-1}. Higher content of phenolic and flavonoids compounds were detected in *in vivo* samples.

Table 4.29: Total phenolic and flavonoids contents of *in vivo* and *in vitro* samples of *Dioscorea alata*.

Sample	Total Phenolic Content	Total Flavanoids Content
In vivo (tuber)	73 mg 100 g^{-1}	32 mg 100 g^{-1}
In vitro (callus)	52 mg 100 g^{-1}	21 mg 100 g^{-1}

* Significant difference at p=0.05 (Independent Samples T-test), n=3

4.7 Antimicrobial Potential in *Dioscorea alata* L. *in vivo* and *in vitro* Samples

4.7.1 Antibacterial activity of *Dioscorea alata* L.

4.7.1.1 Antibacterial Effect of Ethanolic Extract

Four different bacteria, *Escherichia coli*, *Salmonella sp.*, *Staphylococcus aureus* and *Staphylococcus epidermis* were used to screen the possible antibacterial activity of *in vivo* and *in vitro* ethanolic extract of *Dioscorea alata*. Disc diffusion method with definite amount of extracts was used in this study. Chloramphenicol was used as positive control and ethanol 95% as negative control. Table 4.30 showed antibacterial activity of ethanolic extract of *Dioscorea alata*. Clear inhibition zones surrounding the disk of extract indicated the strength of extract to inhibit bacteria growth.

E.coli, *Salmonella sp.*, *S.aureus* and *S.epidermis* were very sensitive to chloramphenicol. Larger than 53.00 mm inhibition zones were observed from extracts against *E.coli*, *Salmonella sp*. and *S.epidermis*. Negative control have a slight effect on all bacteria tested. From Table 4.30, all ethanolic extracts showed bigger inhibition zones compared to negative control.

The growth of *E.coli* (Figure 4.30), *Salmonella sp.* (Figure 4.31), *S.aureus* and *S.epidermis* were inhibited effectively by ethanolic tubers, plantlets and callus extracts. Plantlet extract showed the highest antibacterial effect against *E.coli* with inhibition zone of 12.33 ± 0.67 mm. Tuber extract contained more antibacterial activity against *Salmonella sp.*, and *S.aureus* compared to plantlets and callus extract with largest inhibition zone of 18.00 ± 1.00 mm and 18.33 ± 0.88 mm, respectively.

The antibacterial activity of tubers, plantlets and callus were stronger against *S.epidermis*. All samples extract exhibited larger inhibition zones compared to other

bacteria tested. Strong antibacterial activity with the highest inhibition zone (23.00 ± 1.73 mm), was observed from tubers extract and the lowest inhibition zone (10.67 ± 1.14 mm) was detected from callus extract.

Table 4.30: Effect of ethanolic extract of *in vitro* (callus) and *in vivo* (intact plant) samples of *Dioscorea alata* L. on Diameter of inhibition zone (cm) against Bacteria.

Bacteria (10^{10} CFU/ml)	Inhibition Zone (mm)				
	Control positive (chloro-phenicol))	Control negative (methanol)	*In vivo* sample	*In vitro* sample	
			Tuber	Plantlet	Callus
Escherichia coli	59.00±0. 58^{a}	6.33±0.33^{d}	12.00±0.58^{c}	12.33±0.67^{c}	7.00±0.58^{c}
Salmonella sp.	59.00±1.00^{a}	6.33±0.33^{d}	18.00±1.00^{b}	10.33±0.33^{c}	10.00±1.00^{c}
Staphylo-coccus aureus	28.00±1.15^{a}	8.00±0.58^{d}	18.33±0.88^{b}	10.00±0.58^{cd}	9.33±0.67^{cd}
Staphylo-coccus epidermis	53.33±0.67^{a}	7.00±0.58^{de}	23.00±1.73^{b}	12.00±1.52^{c}	10.67±1.14^{cd}

Values are Mean ± Standard Error (SE) included the diameter of filter paper disc (6.00 mm). Means followed by the same letter are not significantly different at the 0.05 level of confidence by one way ANOVA. n=3.

4.7.1.2 Antibacterial effect of aqueous extract

The antibacterial effects of the aqueous extracts of *in vivo* (tubers) and *in vitro* (callus) samples of *Dioscorea alata* evaluated against *Escherichia coli*, *Salmonella sp.*, *Staphylococcus aureus* and *Staphylococcus epidermis* were represented in Table 4.31. From the results shown in Table 4.31, the aqueous extract of the tubers and callus demonstrated considerable inhibitory effect against all the bacteria tested. The growth of *E.coli* was inhibited effectively by tubers extract with inhibition zone 17.33 ± 1.45 mm. Callus extract showed slight effect on *E.coli* growth with 8.00±0.00 mm inhibition zone. Tubers extract showed slightly higher inhibition zone against *Salmonella sp.* (10.00±0.58 mm) compared to callus extract (9.67±0.88 mm). Tubers and callus extracts showed the same effect on *S.aureus* growth with inhibition zone of 11.33±0.58 mm. Tubers and callus extract had a slight effect on *S.epidermis* with inhibition zone of 7.00±0.00 mm and 8.67±1.76 mm, respectively.

Table 4.31: Effect of water extract on *in vitro* (callus) and *in vivo* (intact plant) samples of *Dioscorea alata* L. on Diameter of inhibition zone (cm) against Bacteria.

Bacteria (10^{10} CFU/ml)	Inhibition Zone (mm)	
	In vivo sample (tubers)	*In vitro* sample (callus)
Escherichia coli	17.33±1.45^{b}	8.00±0.58^{d}
Salmonella sp.	10.00±0.58^{c}	9.67±0.88^{c}
Staphylococcus aureus	11.33±0.58^{c}	11.33±0.58^{c}
Staphylococcus epidermis	7.00±0.58^{e}	8.67±1.76^{de}

Values are Mean ± Standard Error (SE) included the diameter of filter paper disc (6.00 mm). Means followed by the same letter are not significantly different at the 0.05 level of confidence by one way ANOVA. n=3.

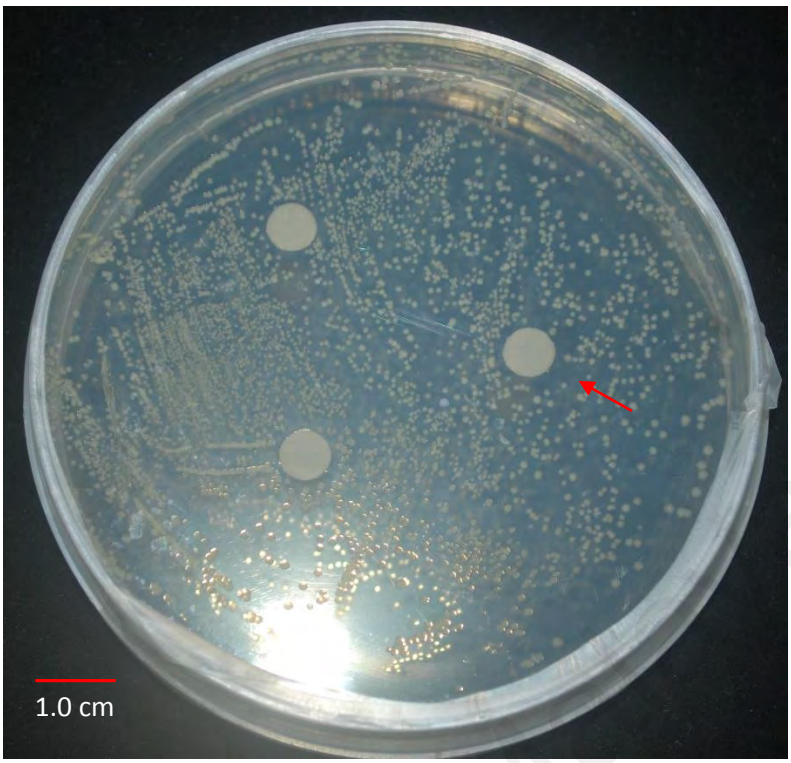


Figure 4.30: Disk diffusion technique. Effect of *Dioscorea alata* tuber ethanolic extract against Gram-negative bacteria *E. coli.*

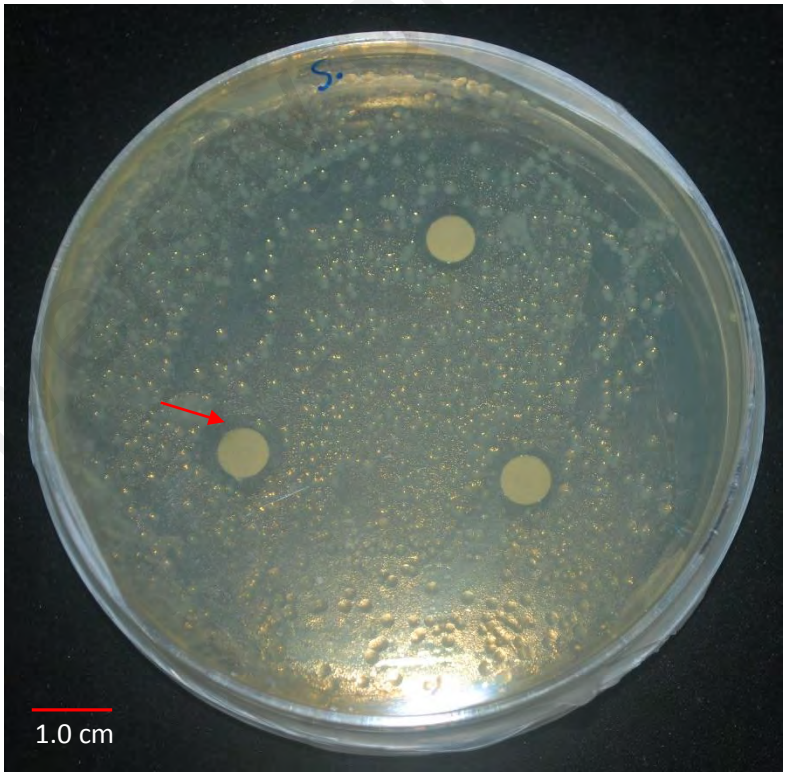


Figure 4.31: Disk diffusion technique. Effect of *Dioscorea alata* tuber ethanolic extract against Gram-negative bacteria *Salmonella sp.*

4.7.2 Antifungal activity of *Dioscorea alata* L.

4.7.2.1 Antifungal Effect of Ethanolic Extract

Four different fungi, *Aspergilus niger*, *Fusarium sp*., *Mucor sp*. and *Penicillium sp*. were used to screen the possible antifungal activity of *in vivo* and *in vitro* samples of *Dioscorea alata*. Disc diffusion method with definite amount of extracts was used in this study. Carbendazim was used as positive control and ethanol 95% as negative control. Clear inhibition zones surrounding the disk of extract indicated the strength of extract to inhibit fungal growth. Table 4.32 showed antifungal activity of *Dioscorea alata* extract.

A.niger, *Fusarium sp*., *Mucor sp*. and *Penicillium sp*. were sensitive to carbendazim. Larger than 20.00 mm inhibition zone was observed from carbendazim against *A.niger*, *Fusarium sp*. and *Mucor sp*. Carbendazim showed the highest antifungal activity against *Penicillium sp*. with inhibition zone of 56.67±0.33 mm. Negative control have no effect againts all bacteria tested except *Penicillium sp*. with small inhibition zone of 6.33 ±0.33 mm .

All the extracts failed to inhibit the growth of *A.niger* (Figure 4.32) and *Fusarium sp*. (Figure 4.33). No inhibition zone was observed from aqueous extract of tubers, plantlet and callus. Extract from *in vivo* (tuber) and *in vitro* (plantlet and callus) inhibited mycelial growth of *Mucor sp.* (Figure 4.34) and *Penicillium sp.* (Figure 4.35). Tubers extract showed the highest inhibition zone against *Mucor sp.* (10.33 ± 0.88 mm) and *Penicillium sp.* (25.00 ± 1.15 mm). The lowest inhibition zone was observed from callus extract against *Mucor sp.* (7.33 ± 0.33 mm) and *Penicillium sp.* (8.00 ± 0.58 mm).

Table 4.32: Effects of ethanolic extract of *in vitro* (callus) and *in vivo* (intact plant) samples of *Dioscorea alata* L. on Diameter of inhibition zone (cm) against fungi.

Fungi (10^{10} CFU/ml)	Inhibition Zone (mm)				
	Control positive (carbendazim)	Control negative (ethanol)	*In vivo* sample	*In vitro* sample	
			Tuber	Leaves	Callus
Aspergilus niger	20.33±2.84[a]	NR	NR	NR	NR
Fusarium sp.	24.00±1.15[a]	NR	NR	NR	NR
Mucor sp.	22.00±1.15[a]	NR	10.33±0.88[b]	7.67±0.67[c]	7.33±0.33[c]
Penicillium sp.	56.67±0.33[a]	6.33±0.33[d]	25.00±1.15[b]	15.67±2.40[c]	8.00±0.58[d]

Values are Mean ± Standard Error (SE) included the diameter of filter paper disc (6.00 mm). Means followed by the same letter are not significantly different at the 0.05 level of confidenceby one way ANOVA. n=3. NR=no response.

4.7.2.2 Antifungal Effect of Aqueous Extract

The aqueous extract of *in vivo* (tubers) and *in vitro* (callus) of *Dioscorea alata* subjected against *Aspergilus nigur, Fusarium sp., Mucor sp.* and *Penilcilium sp*. were represented in Table 4.33. Tubers and callus extracts did not show significant antifungal activity against all fungi tested, hence no inhibition zone was observed.

Table 4.33: Effects of water extract on *in vitro* (callus) and *in vivo* (intact plant) samples of *Dioscorea alata* L. on Diameter of inhibition zone (cm) against fungi.

Fungi (10^{10} CFU/ml)	Inhibition Zone (mm)	
	In vivo sample	*In vitro* sample
Aspergilus niger	NR	NR
Fusarium sp.	NR	NR
Mucor sp.	NR	NR
Penilcillium sp.	NR	NR

Values are Mean ± Standard Error (SE) included the diameter of filter paper disc (6.00 mm). Means followed by the same letter are not significantly different at the 0.05 level of confidence one way ANOVA. n=3. NR=no response.

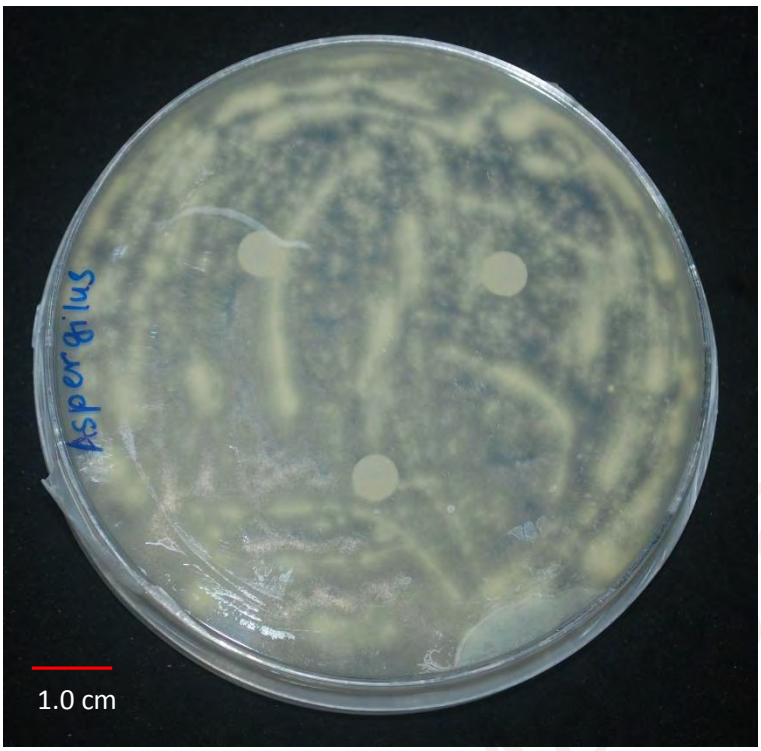


Figure 4.32: Disk diffusion technique. Effect of *Dioscorea alata* tuber ethanolic extract against *A. niger*.

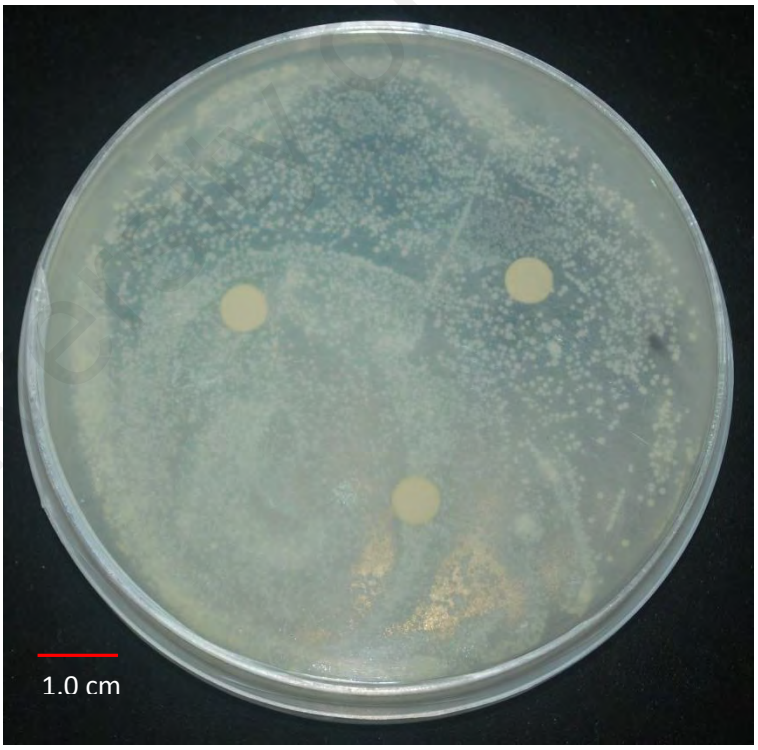


Figure 4.33: Disk diffusion technique. Effect of *Dioscorea alata* tuber ethanolic extract against *Fusarium sp.*

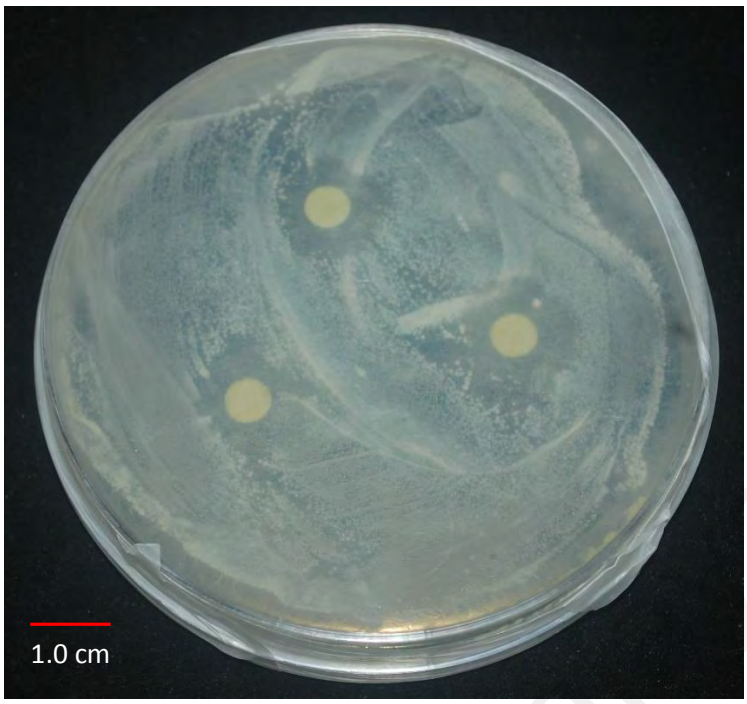


Figure 4.34: Disk diffusion technique. Effect of *Dioscorea alata* tuber ethanolic extract against *Mucor sp.*

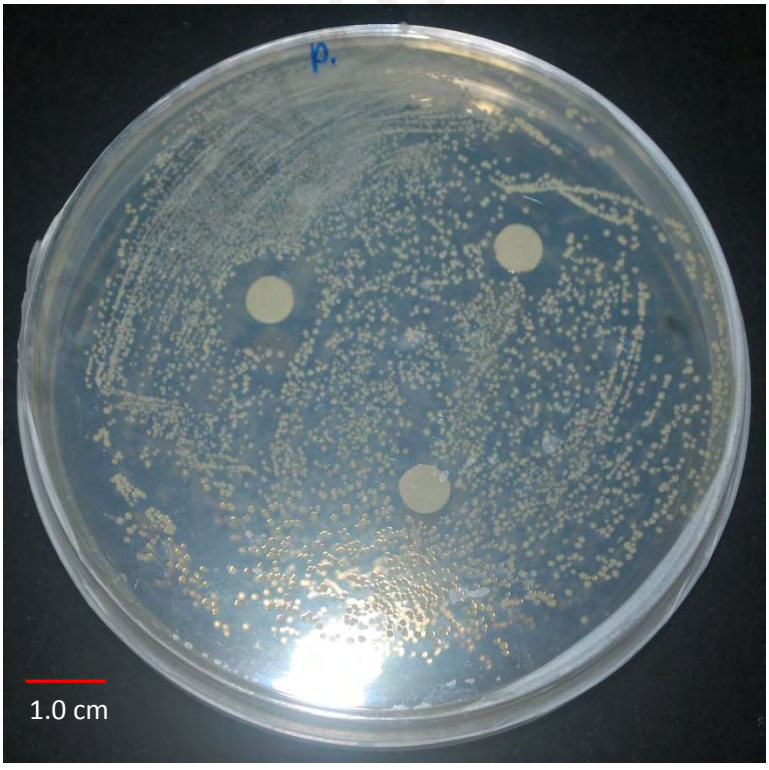


Figure 3.35: Disk diffusion technique. Effect of *Dioscorea alata* tuber ethanolic extract against *Penilcillium sp.*

CHAPTER 5: DISCUSSION

In the present work, tissue culture studies and some biological activities of *Dioscorea alata* L. were investigated. *Dioscorea alata* is well known for their antioxidant activity. This plant is gaining importance in pharmaceutical industries due to their peculiar of various commercial valuable compounds and consumer demands for natural antioxidant and natural food colourant from plant pigments which have potential in health promotion and diseases prevention. Known as 'ubi badak' in Malaysia because of their shape like rhinoceros, this plant receives little attention despite of their good medicinal values with high anthocyanin and antioxidant content. Research, application and access for the tubers of *Dioscorea alata* L. are limited in Malaysia.

Therefore, *in vitro* work was carried out on this species as an alternative method for mass propagation and as a tool to overcome the problems occurring during the conventional propagation. The present tissue culture study was focused on *in vitro* regeneration of complete plantlets of *Dioscorea alata* L. besides callus formation for pigment production. The study was separated into several section. It encompasses the studies on regeneration, microtuberization, coloured callus induction, somatic embryogenesis induction, synthetic seed production, acclimatization of plantlets, anthocyanins accumulation and antimicrobial properties of *Dioscorea alata.* Introduction and literature review of the studies were described in chapter 1 and chapter 2. In chapter 3 section 3.1, studies concerning regeneration and organogenesis of *Dioscorea alata* L. were carried out in order to identify and determine the best concentration and combination of plant growth regulators for optimum microshoots and root formation. Beside, microtubers formation and regeneration from microtubers were also examined.

Like most of *Dioscorea* species, *Dioscorea alata* are highly heterozygous for their cultivars and elites clones and are propagated vegetatively by means of tuber seedlings. Light plays crucial role on tuber germination, conversion and sprouting. Data presented in Table 4.1 showed that seed tubers grew vigorously in light environment compared to in the dark environment. Tubers germinated under light conditions initiated sprouting or shoot growth earlier than those under dark condition. Formation of leaves was observed from tuber seedlings exposed to light. Green purplish leaves were detected from plant grown under direct sunlight and purple leaves were developed from plant grown under fluorescent tube light (Figure 4.1). According to Hamadina et al. (2015), light condition able to support high activity in the peel of the intact tubers may facilitate the progress of the physiological process, such as high crude protein synthesis or metabolism necessary for shoot development. Dark environment is well known for delay sprouting and reduction in the rate of metabolism. This could be attributed to low respiration and evaporative rates in the dark due to lack of light which increases heat energy.

Direct regeneration of *Dioscorea* species could be established from many types of explants. Already, plantlet regeneration *in vitro* for vegetative propagation of important *Dioscorea* species has been achieved using bulbils (Asokan et al., 1983), immature leaves (Kohmura et al., 1995), meristem tips (Maularie et al., 1995a), nodal cutting (Alizadegh et al., 1998; Chen et al., 2003), petioles (Anike et al., 2012) and roots (Twyford & Mantell, 1996). Right choice of explant had great implication for successful organogenesis and regeneration of plants. In the present study, three types of explants were utilized for regeneration of *Dioscorea alata* including leaf, node and stem (internode). The response of leaf, stem and node explants of *Dioscorea alata* when cultured on MS media were presented in Table 4.2. From the observations, leaf and stem explants showed negative response in terms of shoot formation in all treatments. No shoot formation was observed from leaf and stem explants. Node explants showed higher

potential for microshoots formation in comparison to leaf and stem explants. It is well known that node can potentially develop an axillary bud complex and it is the stimulation of axillary bud to develop into a shoot (Figure 4.2). Enlargement and subsequent break of axillary buds was the initial response of node explant. In this study, the axillary bud on the node showed visible growth after 7 days in culture.

Node culture is a method of exploiting the normal ontogenetic route for plant development by lateral meristems. Micropropagation through meristem culture is possible means of virus elimination and produces a large numbers of plants in short span of time. The dependence of cultured node explants on bud initiation and shoot multiplication has already be established for many plant species. This has also been reported in the case of micropropagation of other *Dioscorea sp*. such as *Dioscorea abyssinica* (Martine & Cappadocia, 1991), *Dioscorea batatas* (Koda & Kikuta, 1991), *Dioscorea composite* (Alizadeh et al., 1998), *Dioscorea floribunda* (Sengupta et al., 1984), *Dioscorea oppositifolia* (Behera et al., 2009), *Dioscorea rotundata* (Adeniyi et al., 2008; Anike et al., 2012) and *Dioscorea wightii* (Mahesh et al., 2010). Although in the present study, leaf and stem explants did not produce any shoots in all media tested, leaf and stem cannot be excluded as a promising explants for micropropagation of *Dioscorea alata*. With the right combination and types of plant growth regulators used, leaf and stem explants can be exploited to regenerate shoots. Kohmura et al. (1995) managed to obtain shoots from young leaf explants of *Dioscorea opposita* and Vianna & Mantell, (1989) able to induce shoot formation from callus derived stem explant in *Dioscorea composita*.

In the present study, lower number of shoots (4.33±0.20) was observed from node explants cultured on MS basal medium. Behera et al. (2010), working on the same species found that culture medium without plant growth regulator failed to stimulate the axillary

bud initiation response in the cultured explants even when the cultures were maintained beyond the normal observation periods. However, in several species of *Dioscorea*, node cultures in MS medium without growth regulators had been reported to be an efficient way for shoot multiplication. For example, Chen et al. (2003) successfully induced 74.1 % shoots formation from node explants of *Dioscorea zingiberensis* cultured on MS basal medium without plant growth regulator.

From the results in Table 4.2, it was obvious that shoot formation was affected by the concentrations and combinations of BAP and NAA in the medium. NAA at high concentration (1.5, 2.0 and 3.0 mg/l) in the medium mostly inhibited formation of shoots from node explants. NAA concentration less than 1.0 mg/l had a positive but not significant effect on shoot formation compared to control (MS basal medium). Similar response to NAA treatment was observed in *Dioscorea zingiberensis* (Chen et al., 2003). The average number of shoot formation was lower than control. Almost no shoot formation was detected in MS media fortified with high concentration of auxin. However, this result was contradict to Das et al. (2013) who found that additional of auxin (2.0 mg/l IAA) alone was effective in axillary bud proliferation and shoot formation of *Dioscorea alata*. Additional of BAP at different concentrations into MS basal medium showed significantly impressive response. Of the concentration tested, 1.0 mg/l BAP produced better result with 22. 00 ± 0.43 average number of shoot formation. Average number of shoot formation gradually decreased with the increase of BAP concentration more than 1.0 mg/l BAP. Similar observation was detected in *Dioscorea nipponica* (Chen et al., 2007), on media containing only BAP, the rate of shoot induction increased with increasing plant growth regulator concentration then dropped as the BAP become superoptimal.

It is observed that cytokinin required in optimal quantity for shoot proliferation in many genotypes but addition of low concentration of auxins along with cytokinin triggered shoot proliferation (Sengupta et al., 1984). This is in agreement with observation in the present study. Additional of 0.5 and 1.0 mg/l NAA (auxin) into MS media supplemented with BAP (cytokinins) promoted higher number of shoots formation (Table 4.2). The highest average number of shoot formation, 23.07±0.44 was detected in MS medium supplemented with 1.0 mg/l BAP and 0.5 mg/l NAA. Similar observation was reported in *Spathiphyllum floribundam* (Ramirez-Magon et al., 2001) and *Hovenia dulcis* nodal culture (Echeverrigaray et al., 1998). Similarly Shin et al. (2004) reported that the combination and interaction of BAP and NAA played important role for *in vitro* propagation of node explant for multiple shoot induction. MS medium supplemented with 1.0 mg/l NAA and 0.5-1.0 mg/l BAP was the best concentration for multiple shoot bud induction in *Dioscorea opposita*.

Organogenesis leads to root formation or to the production of unipolar adventitious bud, which develop into shoots that have to be rooted for plantlet formation (Schumann et al., 1995). Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil (Ohyama, 1970). The response and development of rooting from *Dioscorea alata* shoots when subcultured in root induction media were presented in Table 3.3. The results showed, explants successfully produced roots in all media tested including MS basal medium (Figure 4.3). An average of roots (6.63±0.61) were obtained from explants cultured on MS medium without plant growth regulator. Similarly, Poornima & Ravishankar (2007), obtained roots from *Dioscorea oppositifolia* and *Dioscorea pentaphylla* cultured on MS basal medium. However, the current result was contradictory to Das et al. (2013) who found that shoots cultured on MS medium without hormonal supplementation were unable to produce roots and in some shoots rooting was observed but in very negligible amount.

Explants cultured in MS media supplemented with BAP applied singly at all concentration produced low number of roots compared to control. The increases of BAP concentration decreased the average number of root formation. The lowest average number of root formation, (1.57±0.21) was observed when shoots were cultured on MS medium supplemented with 2.0 mg/l BAP. However, rooting was better in the culture media which had combination of BAP and NAA. High number of root formation (17.27±0.61) was observed in MS medium supplemented with 2.0 mg/l NAA and 0.5 mg/l BAP. Similarly, Belarmini & Rosario, (1991) observed 2.0 mg/l NAA in combination with 0.5 mg/l BAP produced better rooting in *Dioscorea esculenta*.

In the present study, NAA applied singly gave better results for root formation. Increase in NAA concentration increased the average number of root formation. NAA at higher concentration (1.5 and 2.0 mg/l) was the best for inducing root formation. Root formation was the highest, (17.40±0.58) in MS medium supplemented with 2.0 mg/l NAA. This observation is in accordance with the result of Behera et al. (2010) in *Dioscorea alata* and Chen et al. (2003) in *Dioscorea zingiberenensis*, except they were using half strength MS. Behera et al. (2009) also reported that MS supplemented with 2.0 mg/l NAA was found to be the best for root formation. Poornima & Ravishankar (2007) obtained efficient number of roots formation in MS medium supplemented with 2.67 µM NAA.

Apart from microshoots and root, formation of microtubers was also observed in the current study. Complete vigorous plantlets with fibrous roots (Figure 4.3) were transferred into microtubers induction media. Plantlets for microtubers induction were maintained in culture room at 16 hours light and 8 hours dark. Jean & Cappodial (1991) indicated that 16 and 24 hours of photoperiods was favourable to produce highest number of microtubers whereas 8 hours photoperiod was effective for larger microtubers. In this

study 30g of sucrose was used in microtuberization media. According to Behera et al. (2009) the highest number of microtubers were obtained on MS medium supplemented with 30g/l sucrose.

In the present study, microtubers generally originated from the axils of basal nodes below the surface of tuberization medium. Microtubers were formed from complete plantlets after 6 months in culture. Microtubers were produced predominantly at basal nodal position on plantlets below culture medium. According to Mantel (1987), microtubers developed from primary nodal complexes (PNC). In the leaf axils of old nodes, there were two axillary buds and shoot primodium, which also later developed into axillary buds. A primary nodal complexes preceded by meristematic PNC-initial is developed at the base of the first form axillary bud. This PNC-initial has capacity for multiple bud production, roots and a tuberous storage organ (Wickham et al., 1982). Thus, after 8 to 10 months microtubers occasionally developed at nodal positions above culture media. The microtubers produced in the basal node above culture medium were small relative to the size of the basal tubers (Figure 4.4).

The effect of different combinations and concentrations of plant growth regulators on microtuberization were identified. The percentage of plantlets produced microtubers, the number of microtubers per plantlet, their length and fresh weight varied according to the concentration of plant growth regulators. Data presented in Table 4.4 indicated that MS medium supplemented with 1.0 mg/l NAA and 1.0 mg/l BAP, was the best medium for microtubers formation with highest mean number of microtubers. According to Pan (2001), NAA could regulate not only vegetative growth but also organ growth whereas BAP facilitated cell division and sprouting. Chen et al. (2007) observed similar result, microtuber of *Dioscorea nipponica* was obtained on MS media supplemented with BAP and NAA. Ng & Mantell (1996) found that NAA enhanced microtuber production in

Dioscorea rotundata. Chen et al. (2007) also stated that synergism of BAP and NAA in their proper concentration is extremely favourable for the tissue culture of *Dioscorea nipponica*.

MS media supplemented with Kin (1.0-2.0 mg/l) have less number of microtubers formation compared to control. This is in agreement with the work of Mantel & Hugo (1989), who reported that Kin inhibited microtuber induction and growth. Comparatively, BAP is more effective than Kin. Indeed the percentage of plantlets produced microtubers with BAP at all concentrations were higher than that of Kin. With concentration of 1.0 mg/l, high percentage of microtubers, 60.00±0.00 and 40.00±0.00 % were obtained, respectively with BAP and Kin. The same result were also reported on the same species by Fotso et al. (2013) but with higher percentage of microtubers formation (72.2%) in BAP then (12.1%) in Kin. Similar results were reported in different species of *Dioscorea* (Forsyth & Van Staden, 1984; Kadota & Nimii, 2004) where BAP was more effective than Kin for microtubers induction.

In vitro tuberization or microtuberisation has been an effective mean for international germplasm distribution or exchange as well as for the propagation of planting materials. Microtubers may provide feasible alternatives to *in vitro* grown plantlets as a mean of germplasm exchange because they are more tolerant to light and temperature variation and less prone to damage than cultured shoots. Microtubers can be stored for long period without losing their viability and field establishment is relatively easy and inexpensive (Ng, 1988).

Regeneration from microtubers was also observed in this study. Microtubers obtained were subcultured and induced to produce complete plantlets with shoot and root. The response of microtubers when cultured on MS media were presented in Table 4.5. Based

on the results, more than 50 % of microtubers managed to germinate in all media tested. This result was contradictory to tuber obtained from traditional planting which has a period of about four months after harvest known as dormancy during which tuber losses are incurred in storage but propagation of the planting materials would not be successful. Dormancy is widely assumed to start at or shortly after tuber maturation. Beside, various studies showed field tubers are dormant well before harvest (Craufurd et al., 2001). However, in the present study no dormancy was observed. Sprouting was detected after 2 weeks of culture. Similar result was obtained by Ondo Ovono et al. (2010) where no dormancy was observed from *in vitro* derived microtubers of *Dioscorea cayenensis-D. rotundata* complex cultured. Alizadegh et al. (1998) also observed no dormancy period in *Dioscorea composita* microtubers.

In the current work of microtubers germination, observation were made on the percentage of microtubers germinated, number of shoots and roots per plantlet. Microtubers were successfully germinated with shoot and root (Figure 4.5) in all 16 media tested included MS basal medium (control). A value of 66.67±0.09 % microtubers were successfully germinated in MS basal medium with an average number of shoots, 4.70 ±0.18 and roots, 1.70±0.11. The results obtained showed that, the highest percentage of microtubers differentiating into shoots was obtained on MS media supplemented with BAP and NAA. These results are similar to those reported by Fotso et al. (2013), combinations of BAP and NAA induced the highest percentage of microtubers producing shoots and the highest number of shoots per plantlet. The fact that auxin and cytokinin must be combined to induce the proliferation of shoots in *Dioscorea alata* confirmed the complementary and synergistic actions of these plant growth regulators as had been shown in several species (Yam et al., 1990; Twyford & Mantell, 1996; Jackson 1999)

In chapter 4 section 4.2, callus induction and coloured callus induction of *Dioscorea alata* L. was investigated. Selection of appropriate explants is a critical step for callus induction for any plant species, particularly monocots where cells differentiate rapidly followed by the loss of mitotic and morphogenetic ability (Krishnaraj & Skirvin, 1995). In the present study, all the explants used, leaf, stem and node successfully produced purplish coloured callus after being cultured onto the media (Figure 4.6, 4.7, 4.8 and 4.9). From the observation, callus started to initiate from the excision side or cut surfaces of the explants and after some time the whole explants were covered with callus. According to Haberlandt (1902), induction of callus formation is due to interaction between wound hormones and other plant growth regulators present. Wounding can increase the uptake of nutrients and growth regulators from the media (Peirik & Steegmans, 1975). This occur because excision of explant would cause the destruction of the cells (Yeoman & Aitchison 1977), elimination or reduction the anatomical barrier which would cause mechanical resistance, (Pierik, 1987) and rupturing of the epidermis upon wounding of the explant has exposed underlying tissue (Rahman & Punja, 2005) to the nutrient and growth regulators. It can be considered as a wound response from almost any part of the original plant, both from plant organs and from specific tissue types or cells (Collin & Edwards, 1998). While, according to Thorpe (1980) and Wagley et al. (1987), formation of callus from explants tissues involved the development of progressively more random planes of cell division, less frequent specialization of cells and loss of organized structures.

Table 4.6, 4.7 and 4.8 showed the effects of plant growth regulators on callus induction in leaf, stem and node explants. All types of plant growth regulators treatment (2,4-D alone, NAA alone, Kin alone, 2,4-D with BAP and NAA with BAP) were successful in showing different frequency of callusing. Higher percentage of explant producing callus was observed in MS medium supplemented with auxin (2,4-D and NAA) compared to callus formation in MS medium without plant growth regulator and MS media

supplemented with cytokinin (Kin) alone. Nodular, friable, soft (watery) callus structure and purplish white callus could be observed in the present work.

Different auxin treatments significantly affected the number of explants that produced callus. More frequency of explants produce callus was observed in treatments with 2,4-D compared to NAA. The higher percentage of explants produced callus was accomplished in MS medium supplemented with 2.0 mg/l 2,4-D compared to 2.0 mg/l NAA. Generally, 2,4-D is known to function in stimulating cellular activity and the formation of morphogenic callus (Trifonova et al., 2001). According to Da Silva et al. (2005), 2,4-D is the main synthetic auxin used to induce callogenesis, because one of its main chracteristic in the capacity to efficiently stimulate the cell division in tissue of several plants.

Different types of explants respond differently to auxin treatment. Among the explants tested, node explants showed better response for callus induction compared to leaf and stem explants (Table 4.6, 4.7 and 4.8). For examples, on MS medium supplemented with 2.0 mg/l BAP, the percentage of explant produced callus in node was 96.67±0.00 compared to leaf, 93.33±0.05. Similar observation has been reported in *Dioscorea rotundata* (Manoharan et al., 2016). However, the percentage of stem explant produced callus as high as node explant (96.67±0.03). This result was supported by Nagasawa & Finer (1989) who found that stem segment of *Dioscorea opposita* were more responsive compared to node and leaf explants.

Additional of cytokinin (BAP) to the callus induction medium showed positive effect on frequency of callus formation from all explants tested. The highest percentage of explants produced callus was obtained from node explants cultured on MS medium supplemented with 2.0 mg/l 2,4-D combined with 1.0 mg/l BAP. Combinations of 2,4-D

and BAP were more favourable than 2,4-D applied singly. However, BAP used as the sole regulator could not induce formation of callus. A similar phenomenon has been reported for *Dioscorea zingiberensis* (Shu et al., 2005).Two types of regeneration systems were tested for better callus production. Results in Table 4.4 indicated that liquid media was the best for coloured callus production in *Dioscorea alata*. This might be due to better aeration produced in liquid media.

During micropropagation process, the exudation of phenol is very common and is often influence the result of the experiment. The three explant types of *Dioscorea alata* showed darkening of the wounded tissues immediately after excision and exuded purplish brown substance into the culture media. The addition of activated charcoal in tissue culture media is a recognize practice and its influence in growth and development maybe attributed mainly to the adsorption of inhibitory substances in the culture medium (Theander & Nelson, 1988; Weatherland et al., 1979), drastic decrease in the phenolic oxidation or brown exudate accumulation (Liu, 1993) alteration of medium pH to an optimum level for morphogenesis (Owen et al., 1991) and establishment of darkened environment in medium and hence stimulate soil conditions (Dumas & Monteuuis, 1995). Activated charcoal (AC) is composed of carbon arranged in a quasigraphitic form in small particle size. It is a porous and tasteless material and is distinguished from elementary carbon by removal of all noncarbon purities and the oxidation of carbon saurce. Activated charcoal has a very fine network of pores, an extraordinary large surface area and volume that gives it a unique adsorption capacity (Baker et al., 1992).

In the present study, activated charcoal was added into the media because of its capability to absorb phenolic compounds that excreted from the cutting explants. The addition of 0.1 % activated charcoal effectively eliminated the accumulation of phenolic compounds in the media. No media browning and contamination were observed. Similar

result was obtained by Poornima & Ravishankar (2007), whereby, it was found that the additional of activated charcoal in the culture medium had successfully reduced phenolic exudation, and better growth response have been associated with addition of activated charcoal. According to Weatherhead et al. (1978), better growth responses of plant tissues have been associated with addition of activated charcoal as it removed inhibitory substances from the media.

However, in this study, alongside with no phenolic compound secretion, no response of explants cultured on media supplemented with activated charcoal was observed. The highest frequency of callus formation, only 13% was observed in MS medium supplemented with 0.1 % activated charcoal and 2.0 mg/l 2,4-D with 1.0 mg/l BAP (Table 4.10). This result was a contradict to some other researchers who worked on other *Dioscorea* species. Majority of the reports confirm the positive role of activated charcoal in medium promoting growth and development of plant tissues. For example, in *Dioscorea fordii*, additional of activated charcoal into the culture media managed to induce the formation of microtubers (Yan et al., 2011). Better rooting was observed in *Dioscorea oppositifolia* and *Dioscorea pentaphylla* when activated charcoal was added to the culture medium (Poornima & Ravishankar, 2007).

Even though the effect of activated charcoal on plant growth regulators uptake is still unclear, some researchers believed that activated charcoal may gradually release certain adsorbed products, such as nutrients and plant growth regulators in addition to the release of substances naturally present in activated charcoal which promote plant growth (Johansson & Eriksson, 1977; Johansson et al., 1990). However, according to Han et al. (2005), activated charcoal adsorb not only inhibitory substances accumulating in the culture medium but also auxin and cytokinin present in the media. Activated charcoal has certain preference for polar rather than apolar organics (Thomas, 2008). Similarly greater

absorptive capacity towards aromatic products like phenolics and their oxidates, auxin (IAA, NAA, IBA), cytokinin (BAP. Kin, Zeatin) and other plant growth regulators than olefinic unsaturation product. In the present study, the facts that initiated media for callus induction was supplemented with auxin and cytokinin, might be the reason why there were no or less response of explants cultured after additional of activated charcoal.

The current results suggested that the treatment with activated charcoal almost inhibited callogenesis response in *Dioscorea alata*, attributing to the absorption of growth regulators (2,4-D and BAP) by activated charcoal from the medium. These results were supported by Anna et al. (2007) who observed similar inhibitory impact of activated charcoal on callus formation in *Melaleuca alternifolia* and Lu et at. (2002) working on *Morus latifolia*. The difficulty in using activated charcoal in medium is that in addition to adsorbing unwanted substances, it may also adsorb needed plant growth regulators (Ebert et al., 1993; Nissen & Sutter, 1990), vitamins (Weatherhead et al., 1978; Pan & van Staden, 1998) or metal ions such as Cu^{+2} and Zn^{+2} (Van Winkle et al., 2003).

Myo-inositol is a small molecule that is important in many different developmental and physiological processes in plant cells. Myo-inositol participates in the phosphatidylinositol signaling pathway, auxin storage and transport, phytic acid biosynthesis and cell wall biosynthesis. Myo-inositol is included in most tissue culture media, as its addition is believed to improve plant regeneration, although the basis for its requirement is unknown; equally unknown is why in some cultures do not require myo-inositol or produce excess inositol. According to Hrib et al. (1997), cultured cells may or may not require exogenous myo-inositol, depending on their differentiation stages.

In the current study, results indicated that there was no significant difference for all the treatments, in term of percentage of explants producing callus (Table 4.11). However

maximum callus growth was obtained in culture medium supplemented with additional myo-inositol. Node explants cultured in MS medium supplemented with 2.0 mg/l 2,4-D and 1.0 mg/l BAP added with 0.5 mg/l myo-inositol was the best condition for optimum callus production. The results of this study showed that myo-inositol is of vital importance for growth and maintenance of callus. This result was supported by Wenxing Ye et al. (2016) who did research on 'Transcriptomic profiling analysis of *Arabidopsis thaliana* treated with exogenous myo-inositol'. According to their findings, the expression of gene AtMIOX2 was also upregulated in root and callus of myo-inositol treatment. They also found that exogenous myo-inositol could reduce cell death and maintained normal cell growth and development.

Callus initiation was observed earlier on MS media supplemented with myo-inositol. Callus growth rate was slow without myo-inositol treatment. Exogenous myo-inositol also affect nutrients absorption. Wood & Braun (1961) showed that the synthesis of myo-inositol can be initiated or inhibited by merely changing the inorganic constituent of the growth medium. Increasing levels of myo-inositol promote rapid callus formation and callus growth. Same result was reported by Karl & Lidjia (1977), normal green ash callus tissue grew exponentially on medium containing myo-inositol and no callus observed on a medium lacking myo-inositol. The above observations suggest that cells require myo-inositol, which may be taken up from the medium or it may be synthesized by the myo-inositol-1-phosphate synthase.

Although plant cells take up and subsequent metabolize myo-inositol, when these cell are deprived of myo-inositol there is often no deleterious effect. This is because plant cell have the capacity to synthesize myo-inositol from D-glucose (Loewus & Kelly, 1962) via the enzyme L-myo-inositol 1-phosphate synthase, which utilized D-glucose 6-phosphate as substrate (Funkhouser & Loewus, 1975; Loewus & Loewus, 1983). To reduce the

level of endogenous myo-inositol it may not be sufficient solely to remove the extracellular myo-inositol but de novo synthesis of myo-inositol may also need to be inhibited. Donahua et al. (2010) suggested exogenous myo-inositol could rescue spontaneous cell death and lesions on leaves in *Arabidopsis atmis1 mutant*.

Light is a very important physical factor for callus induction, cell growth and production of plant secondary metabolites. Light controls plant growth and development mainly in two ways: photosynthesis and photomorphogenesis. Under normal tissue culture condition, callus culture are known to develop chloroplast in the light, carry out photosynthesis and evolve oxygen (Bergmann et al., 1966; Neumann et al., 1973). However, the presence of sucrose in the culture medium inhibits both chlorophyll synthesis and carbon fixation in callus culture (Seibert et al., 1980). Thus, callus are not photosynthetically efficient and generally not autotrophic.

Production of coloured callus also influenced by light. However, the degree responses to light is depend on cell types and plant species. In *Dioscorea alata*, percentage of explants produced callus was found to be independent from the various time of light exposure. Explants managed to produce callus in all treatments, no significant difference observed on average callus formation frequency (Table 4.12) but explants cultured under light condition gave slightly larger average size in callus than those cultured under dark condition as observed generally in the current work. In the present study, though light has no effect on frequency of callus formation, however different colours of callus were obtained from different photoperiod exposed (Figure 4.10).

A number of researchers mentioned light had significant effect on callus growth and morphogenesis, the inhibition of axillary shoot proliferation and the induction of specific enzyme activity that is linked with the formation of some flavonoid glycoside secondary

product (George et al., 2008) such as anthocyanin. It is well known that anthocyanin properties including colour expression are highly influenced by anthocyanin structure, light and pH. Triggering of anthocyanin synthesis by light has been demonstrated in callus from several plant species (Strickland et al., 1972). Further experiments of anthocyanin content in coloured callus exposed to light at different photoperiod were discussed in Chapter 4 section 4.6.3.

Callus can be induced for organogenesis and somatic embryogenesis pathways. Somatic embryogenesis is a multi-step regeneration process starting with the formation of proembryogenic masses, followed by somatic embryo formation, maturation, desiccation and plant regeneration (Von Arnold et al., 2002). This process is the best exemplifies the concept of totipotency, that all normal living cells possess the potential to regenerate the entire organism. Somatic embryo can provide a useful model to study embryo development in plant (Zimmerman, 1993). Once the induction of an embryogenic state is complete, the mechanisms of pattern formation that lead to the zygotic embryo are common to all other form of embryogenesis (Mordhorst et al., 1997). Thus, somatic and zygotic embryos share similar gross ontogenies, with typically passing through globular, heart shape and torpedo shape stages in dicots or globular scutellar (trasition) and coleoptilar stages in monocots (Gray et al., 1995; Toonen & de Vries, 1996).

Somatic embryogenesis is a very valuable tool for achieving a wide range of objectives, from basic biochemical, physiological and morphological studies, to the development of technologies with a high degree of practical applications (Victor, 2001). According to Haccius (1978), somatic embryogenesis has been considered to be a distinct developmental pathway different from shoot or root organogenesis, in which a single cell gives rise to a structure containing bipolar meristem and with no direct vascular connection to the maternal tissue. The mass propagation of plants through multiplication

of embryogenic propagules is the most commercially attractive application of somatic embryogenesis (Merkle et al., 1990). Somatic embryogenesis potentially offers a promising system for plant regeneration because of the high proliferation capacity and the probable single cell origin, which may avoid the risk of chimeric plants and facilitate the application for mutant selection and recombinant DNA technology (Luo et al., 1999; Ponsamuel et al., 1996; Roberts et al., 1995; Stefaniak, 1994).

Thus in chapter 4 section 4.3, experiments on production of embryogenic callus and formation of somatic embryos were conducted. The choice of explants has a great impact on the callus induction and success of somatic embryo formation. According to Thorpe, (1995), the relative responsiveness of various immature tissues seems to be species-specific. For example, cotyledons were more embryogenic than hypocotyls in cucumber (Chee, 1990) while in cotton hypocotyls produced more embryos than seeds or cotyledons (Trolinder & Chen, 1989). Research has been done to observe embryogenic response among young tissue (Punja et al., 1990; Chee, 1990), mature vegetative tissue (Willian & Maheswaran, 1986) and floral tissue (Carman, 1990). According to Carman (1990), floral tissue (ovaries, pedicels, peduncles, buds and inflorescences) may be embryogenic because of their developmental proximity to embryogenesis *in vivo*.

For *Dioscorea* sp, explant such as axillary bud (Manoharan et al., 2016), node segment (Belarmino & Gonzales, 2008), root segment (Twyford & Mantell, 1996), stem segment (Nagasawa & Finer, 1989) and zygotic embryos (Osifo, 1988) have been demonstrated to induce somatic embryo formation. Based on these earlier reports and observations made in Chapter 4 section 4.2, node segment was utilized as the explant for embryogenic callus induction and somatic embryo formation in *Dioscorea alata* L. From the observation, among the three types of explant (leaf, stem and node) used, the node explant produced more purplish nodular embryogenic callus in shorter time (2 weeks). In contrast,

stem explant take longer time (5 weeks) to form callus and leaf explant produced purplish soft fine non-embryogenic callus that were highly vacuolated.

In the present study, node explant was cultured on MS media supplemented with 0.5 to 3.0 mg/l NAA and 0.5 to 3.0 mg/l 2,4-D to induce embryogenic callus formation. Results showed that high percentage of explants produced callus were detected in all media tested, except in MS devoid auxin. No callus formation was observed. Low concentration of NAA (0.5 mg/l) was the least favourable NAA concentration for callus formation. However, NAA at medium concentrations (2.0 and 2.5 mg/l) are the best for callus formation with 90.00 % explants produced callus (Table 4.13).

Culture media composition is very important to induce embryogenic callus. Results obtained in the present study of *Dioscorea alata* showed, medium concentrations of auxin was essential to enhance embryogenic callus production with 2,4-D significantly better than NAA. Luo et al. (1999) reported similar observation, induction of embryogenic callus was usually promoted by a relatively high concentration of auxins especially 2, 4-D. According to Van der Valk et al. (1992), 2,4-D was predominantly used for the induction of embryogenic callus in monocotyledonous plants. Similar results also reported by Nagarajan et al. (1986), Meijer & Brown (1987), Nolan et al. (1989), Shri & Daris (1992), Carman (1990) and Ammirato (1987).

The callus obtained from node explants was further tested for their embryogenic nature by using double staining technique (Gupta & Durzan, 1987) and observed under a light microscope. Observation under a light microscope, proved that purplish white callus obtained from node explants was embryogenic callus. Embryogenic callus cells were stained red with acetocarmine consists of large nuclei and dense cytoplasm (Figure 4.13a

and Figure 4.13b) while non-embryogenic callus cells were stained blue with Evan's blue and have very small nuclei (Figure 4.13c).

Embryogenic callus would develop into somatic embryos under suitable conditions. In order to stimulate somatic embryo development, embryogenic callus obtained from node explants were then transferred onto MS media supplemented with NAA alone, 2,4-D alone, BAP alone, NAA combined with BAP and 2,4-D combined with BAP prepared in solid and liquid media. In the present study, the transfer of embryogenic callus to somatic embryogenic induction media, successfully induced the formation and maturation of somatic embryos. After subculture, some of the callus become compact, globular and turn white with a little brown indicating they were embryogenic in nature. The non-embryogenic callus remained friable throughout the culture.

When the callus obtained were transferred to somatic embryogenesis induction media, internal cell divisions led to the formation of proembryo (Figure 4.11 and 4.12), distinct from each other in the callus at the beginning of the developmental phase of somatic embryos. Somatic embryos were easily dislodged from each other and the parent callus. According to Hicks (1980) in somatic embryogenesis, a plant with both roots and shoots axes arises from actively dividing cells, but does not form any direct vascular connections with the original tissue, while in organogenesis vascular system of shoots and roots are often connected to the parent tissue. This probably indicates the single cell origin of embryos. Unicellular origin of somatic embryogenesis obtained in the present study may allow the production of non-chimeric plants of *Dioscorea alata*.

After 2 months in culture, round structure of globular stage could be observed (Figure 4.14) and subsequently developed into scutellar and then coleoptilar stages (Figure 4.15). Based on Figure 4.16, asynchronous somatic embryos formation and development were

observed in this species. Different stages of somatic embryo development could be observed within the same treatment and replication unit. Asynchronous was observed also in somatic embryogenesis callus culture of Kampung Royal Poinciana (Myers & Vendrame, 2004), *Astragalus adsurgens* Pall (Luo et al., 1999), *Azadirachta indica* A. Juss (Medha et al., 1993) and *Dioscorea composita* and *Dioscorea cayenensis* (Viana & Mantell, 1989).

Many factors including choice of growth regulators, choice of explants and culture medium composition are responsible for successful of somatic embryogenesis. Based on Table 4.14, results indicated that embryogenic callus subcultured in liquid media produced more number of somatic embryos compared to solid media with the same plant growth regulator treatment. Liquid medium seems to be more effective which could be due to better aeration. Many researchers utilized liquid suspension culture for rapid development of somatic embryos. Winkelmann et al. (1998) and Hohe et al. (2001) used cell suspension culture for large scale production of *Cyclamen* somatic embryos. Atanassov & Brown (1984) and Mc Kersie et al. (1989) used suspension cultures to induce somatic embryos from *Medicago sativa* L.

Plant growth regulators have profoundly influenced the embryogenesis process in plant species and suitable medium composition should be worked out for embryo induction, development, maturation and conversion. In the present study, no further growth observed from embryogenic callus subcultured onto MS basal medium. Embryogenic callus were observed dehydrated after 2 weeks in the medium. These results were in contrast with many herbaceous monocots species such as cucumber (Raharjo & Punja, 1994), melon (Gray et al., 1993), squash (Chee, 1992) and strawberry (Wang et al., 1984). According to their report, embryogenic callus subsequently gave rise to somatic embryos when growth regulators were removed. However, according to

Laksmanan & Taji (2000), the removal of plant growth regulators could be critical for somatic embryo differentiation and maturation. This finding was in agreement with the results of this study.

No somatic embryo formation was observed on MS media devoid of auxin (Table 4.14). MS media supplemented with auxin showed better response for somatic embryos formation with 2,4-D better than NAA at the same concentration. Auxin plays a critical role in the reactivation of the cell cycle and the initiation of the reactivation of the cell cycle and the initiation of the embryo formation. According to Feher et al. (2003), auxins are considered a major factor evoking embryogenic competence in somatic tissues to induce somatic embryogenesis. Application of high concentration of 2,4-D in the culture medium itself is a stress signal, since embryogenic induction requires the use of a physiological auxin concentration that inhibit callus growth.

In the present study, MS medium supplemented with BAP at concentration of 0.5 to 2.0 mg/l in the medium had inhibited the formation of callus in *Dioscorea alata*. However, addition of 2,4-D into the media at concentrations of 0.5 to 2.0 mg/l successfully produced more somatic embryos comparable to embryo produced in MS supplemented with 2,4-D alone. MS medium supplemented with 2.0 mg/l 2-4,D and 1.0 mg/l BAP produced the highest average number of somatic embryos, (23.27 ± 0.14). This results showed that, combination of auxin and cyctokinin played an important role in somatic embryos production of *Dioscorea alata* L. Similar result was observed by Ntui et al. (2010) where in *Digitaria exilis*, somatic embryogenesis was observed higher in medium containing 2,4-D supplemented with BAP compared to medium supplemented with 2,4-D alone This observation was in agreement with Trinh et al. (1998) who found that MS supplemented with 2,4-D and BAP promoted and enhanced embryogenic callus production and subsequent embryo differentiation in *Medicado truncatula*. Similar needs

for somatic embryo formation also reported for other species such as *Coffea canephora* (Hatanaka et al., 1991), *Coronilla varia* (Moyer & Gustine, 1984), *Eleusina caracana* (Eapen & George, 1989) and *Thevetia peruviana* (Kumar, 1992).

Somatic embryos were transferred to germination media to encourage embryos maturation and germination. The embryos at globular stages underwent sequential development stages then germinated one month after transferred in germination media. The first sign of somatic embryo maturation was the formation of cotyledonary collar. Hook type of germination was observed from the cotyledonary collar. Tiny leaves like hook started to emerge from the opening of the collar (Figure 4.17). The present results are in agreement with previous reports described for somatic embryogenesis in *Dioscorea* sp. Twyford & Mantel (1996) also observed the cotyledonary collar and hook type of germination in *Dioscorea alata*. Ammirato (1984), observed the formation of cotyledonary collar surrounding the first leaf primodium in each somatic embryo in *Dioscorea floribunda*. Monoharan et al. (2016) observed cotyledonary collar, fan-shaped and hook types of germination in *Dioscorea rotundata*.

Several concentrations and combinations of plant growth regulators were tested for somatic embryos maturation and germination in *Dioscorea alata* L. In the present study, the embryos were successfully germinated on MS medium without growth hormone. MS medium without growth regulators is a typical medium for somatic embryo maturation and germination in many plants includes Dioscoreacea, *Dioscorea alata* (Belarmino & Gonzales, 2008), *Dioscorea rotundata* (Monoharan et al., 2016), *Dioscorea opposita* (Nagasawa & Finer, 1989) and other monocots species like *Sporobolus virginicus* (Straub et al., 1992), *Scirpus robustus* (Wang et al., 2004). However, the germination rate was low (23.33±0.08) in MS medium without growth hormone due to slow embryos maturation development. Somatic embryos showed weak growth with small number of

shoots formation (7.00±0.31). These observations also in agreement with those obtained by Belarmino & Gonzales (2008). Wang et al. (2004) obtained the same result when germinated somatic embryos of monocot *Scirpus robustus* in MS medium, weak shoot growth with both small shoots numbers and shoots no more than 1 cm in height was obtained.

Hence, BAP was added into the germination media for better germination rate. Based on results in Table 4.15 addition of BAP in the media, improved the overall germination of somatic embryos. Higher percentage of germination was observed in all media fortified with BAP singly at all concentrations compared to in MS basal medium. The highest percentage, (60.00±0.09) of embryos germination was observed on MS medium supplemented with 1.0 mg/l BAP. The stimulation of germination by BAP also reported in other *Dioscorea* sp. Nagasawa & Finer (1989) found that 50 % of the total embryos germinated on MS medium supplemented with 3.0mg/l BAP. Wang et al. (2004), observed increasing of shoot formation in *Scirpus robustus* from 8 to 35 number of shoots.

In the present study, additional of NAA to the BAP supplemented media significantly decreased the percentage of embryos germination and number of generated shoots and roots. This result was contradictory to those obtained by Medha et al. (1993), where they manage to germinate and enlarged 60 to 70% of somatic embryos of neem on MS medium containing 2.0 mg/l BAP and 0.5 mg/l IAA after 20 to 30 days. Formation of roots was detected from globular stage of somatic embryos on MS media supplemented with BAP in combination with NAA (Figure 4.18), however low number of roots formation was observed. Usually, shoot were regenerated from somatic embryos.

Another method of micropropagation is synthetic seeds production. Hence, Chapter 4 section 4.4 discussed the production of synthetic seeds from *Dioscorea alata* L.

propagules. There is currently much interest in creating synthetic seeds possessing outer protective coatings, thereby bestowing the vegetative tissues with the storage and handling qualities of natural seed. Synthetic seed technology could be useful in conservation of clonal germplasm of elite and endangered plants in near future, with development of appropriate storage technique (Niino & Sakai, 1992; Na & Kondo, 1996). In addition, synthetic seed technology could be the exchange of axenic plant material between laboratories (Piccioni & Standardi, 1995; Ara et al., 1999).

Murashige (1977) was the first to propose the idea of synthetic seed by encapsulated somatic embryos. Four delivery systems have been proposed. 1) fluid drilling of somatic embryos, 2) encapsulation of somatic embryos in an alginate gel, 3) desiccation of somatic embryos and 4) desiccation of encapsulated somatic embryos using water resin. However, there were several limitations in the production of synthetic by encapsulated somatic embryos. The lack of synchrony of somatic embryos is perhaps the major restriction in the development of synthetic seed. Synthetic seed technology requires the large numbers of high quality somatic embryos with synchronous maturation. The production of synthetic seed by encapsulating vegetative parts such as shoot tips, shoot primordial or axillary buds had been used in recent years are more suitable alternative to somatic embryos. Therefore, in this experiment, encapsulation of vegetative parts such as shoot tips, node and stem segments in alginate gel solution were carried out.

Alginate is frequently selected as an encapsulation matrix for synthetic seed technology because of its moderate viscosity and low spin ability of solution, low toxicity for propagules (shoot tips, nodes and stems) and quick gelation, low cost and bio-compatibility characteristics. In the present experiment, alginate was chosen because it enhanced capsule formation and also the rigidity of alginate beads provided better protection to the encased propagules against mechanical injury. The major principle

involves in the alginate encapsulation process is that the sodium alginate droplets containing the explants, when drop into the Calcium chloride dehydrate ($CaCl_2.2H_2O$) solution form firm and round beads due to ion exchange between the Na^+ in Sodium alginate with Ca^{2+} in Calcium chloride dehydrate solution. The hardness or rigidity of the capsule mainly depends upon the number of sodium ions exchange with calcium ions. Hence, the concentration of the two gelling agents and complexation time should be optimized for the formation of the capsule with optimum bead hardness and rigidity.

In the current work, four concentrations of Sodium alginate (2, 3, 4 and 5 %) and four concentration of Calcium chloride dehydrate (25, 50, 75 and 100 mM) were tested for ideal bead formation with 30 min complexation time. The effect of different concentrations of Sodium alginate and Calcium chloride dehydrate on synthetic seeds formation was presented in Table 4.16. It was found that 3.0 % Sodium alginate complexation with 75 and 100 mM Calcium chloride dehydrate gave optimal bead hardness and rigidity for the production of viable synthetic seeds (Figure 4.19). This particular results obtained maybe due to an optimal ion exchange between Na^+ and Ca^{2+}. This result is in agreement with many other reports on synthetic seed production such as *African violet* (Daud et al., 2008), and *Brassica oleracea* (Poon et al., 2012). At lower concentration (2.0 %) sodium alginate solution became unsuitable for encapsulation possibly because of a reduction in its gelling ability following exposure to high temperature during autoclaving (Larking et al., 1988). At a higher concentration of sodium alginate solution (5.0 %), beads were isodiametric but hard enough to cause considerably delay in sprouting. Similar observations were also made in *Solanum tuberosum* (Sarkar & Naik, 1997) and *Withania somnifera* (Singh et al., 2006).

Synthetic seeds were inoculated on MS basal media solid and liquid for germination (Figure 4.20). Sprouting of synthetic seeds were observed from all the three types

encapsulated propagules in both liquid and solid media. However, of the two media tested for conversion (ability of synthetic seeds to grow into a plantlets), best response for both shoot and roots emergence was recorded from encapsulated propagules in MS liquid medium. Similar observation was also made in *Psidium guajava* (Rai et al., 2008). Synthetic seed germinated on MS liquid media, could break the bead earlier than synthetic seed germinated in MS solid medium, however, significant delay in conversion was observed from synthetic seeds germinated in liquid media.

Based on results in Tables 4.17, 4.18 and 4.19, same percentage of germination rate was observed in synthetic seeds cultured on solid or liquid media. The comparison of solid and liquid media in this experiment showed, that the two culture systems did not differ regarding the germination rate. However, it can be explained by the length of the culture period. In this experiment, synthetic seed cultured in solid media take longer time to germinate compared to synthetic seeds cultured in liquid media. This point the fact that liquid cultures result in much faster growth, but the germination potential is the same as for the cultures on solid medium.

Tables 4.17, 4.18 and 4.19 also showed that, synthetic seeds sprouting was affected by propagules type. Encapsulated shoot tips and node segments germinated earlier and the frequency of germination was also higher in all treatments compared to encapsulated stems. According to Ballester et al. (1997) shoot tip explants were more responsive than other non-embryogenic propagules because of greater mitotic activity in the meristem. Encapsulated node explants also germinated earlier than stem explants probably because node segments contained the axillary buds (existing meristems) for shoot tips formation.

From Tables 4.17, 4.18 and 4.19, it was observed that concentrations of sodium alginate solution and calcium chloride solution can influence the frequency of synthetic

seeds germination. Explants encapsulated with lower concentrations of sodium alginate solution and calcium chloride solution (2% sodium alginate and 25, 50, 75 and 100 mM Calcium chloride dehydrate, 3% sodium alginate and 25, 50 mM Calcium chloride solution and 4% sodium alginate and 25 mM Calcium chloride solution) managed to germinate earlier than explants encapsulated with higher concentration. Although, these synthetic seed germinated earlier, it was difficult to handle and the shape was not favourable. It was concluded that these concentration of sodium alginate solution and calcium chloride solution was not suitable for synthetic seeds formation for *Dioscorea alata*.

Encapsulated explants with 3% sodium alginate hardened in 100 mM calcium chloride dehydrate was chosen as the best encapsulation medium from previous experiment with optimal bead hardness and rigidity for the production of viable synthetic seeds. Cracking of beads was first observed with outgrowth of shoot from shoot tip explants encapsulated with 3% sodium alginate and 100 mM calcium chloride dehydrate after 7 days in culture followed with node propagules, 10 days after cultured. Figure 4.21 showed the development of synthetic seeds (encapsulated node propagules) of *Dioscorea alata* into complete plantlets. One step germination i.e both shoot and root formation were detected. Encapsulated stems started to germinate after 14 days in culture without shoot or root emergence. Formation of purplish white callus was detected.

Shoot tips, node and stem propagules have no seed coats and endosperms that provide protection and nutrition in developing seeds. Therefore, addition of nutrient and plant growth regulators to the encapsulation matrix is desired, which serves as an artificial endosperm. Tables 4.20, 4.21 and 4.22 showed the additional of 1.0 mg/l BAP singly and 1.0 mg/l BAP combined with 2.0 mg/l 2,4-D to the encapsulation matrix resulted in increase the efficiency of germination and viability of synthetic seeds. Encapsulated shoot

tips, node segments and stems manage to germinate earlier in culture media with plant growth regulators compared to encapsulated propagules in sodium alginate without plant growth regulator. Node segments encapsulated with 3.0 % sodium alginate supplemented with 1.0 mg/l BAP and 1.0 mg/l BAP combination with 2.0 mg/l 2,4-D, germinated 3 days earlier compared to node segments encapsulated with 3.0 % sodium alginate without plant growth regulator (Table 4.20). Similar observations were recorded for encapsulated shoot tips (Table 4.21) and stem (Table 4.22) propagules.

Higher germination rate was also observed from propagules encapsulated with sodium alginate solution supplemented with plant growth regulators. The percentage of germination also increased with the addition of plant growth regulators into the encapsulation media. Encapsulated shoot tips with plant growth regulators successfully germinated a higher rate (96.67 ± 0.03 %) compared to 90.00 ± 0.06 % in control (encapsulated shoot tips without plant growth regulators). 100 ± 0.00 % of encapsulated node segments managed to germinate in liquid and solid MS media while, 56.67 ± 0.09 % encapsulated stem explant with 3% Sodium alginate hardened in 75 mM Calcium chloride dehydrate supplemented with 1.0 mg/l BAP singly or in combination with 2.0 mg/l 2,4-D and 53.33 ± 0.09 % encapsulated shoot tips with 3% Sodium alginate hardened in 100 mM Calcium chloride dehydrate successfully germinated compared to 46.67 ± 0.09[a] % in control. The same percentage of germination rate was observed from synthetic seeds cultured on solid or liquid media.

A desirable feature of the synthetic seeds is their ability to retain viability in terms of germination potential even after a considerable period of storage. Low temperature and high humidity were essential conditions for retention of viability and thus for storage of encapsulated propagules. Earlier, low temperature storage of encapsulated propagules has been demonstrated by several researcher (Naik & Chand, 2006; Rai et al., 2008).

Therefore, in the present study, encapsulated shoot tips, node segments and stem explants were stored at 4.0 ºC and their germination potential were observed. The effect of storage periods on germination of synthetic seed is presented in Table 4.23. A decreasing of germination rate was observed from synthetic seeds of *Dioscorea alata* stored at 4.0 ºC. The results showed, the number of synthetic seeds germinated and percentage of germination were decreased with time prolong in the cold storage.

The longer duration of cold storage of synthetic seeds caused significant reduction in plantlets recovery. In the present study, the viability of synthetic seeds had fallen drastically from 100% to 3.33% after 30 days of storage. Similarly, Naik & Chand (2006) observed the conversion frequency of encapsulated node segments of *Punica granatum* also declined markedly following storage at low temperature. The declined in the regeneration frequency among the stored encapsulated propagules might be due to the inhibited respiration of plant tissue by the alginate coating and accumulation of metabolite waste in the alginate capsules during the long storage.

After 45 days at low temperature storage, synthetic seed of *Dioscorea alata* lost their viability completely. It is assumed that any rate of decline in the germination frequency observed among encapsulated propagules stored at low temperature may have resulted because of inhibited respiration of plant tissue perhaps due to alginate cover (Redenbaugh et al., 1987) and oxygen deficiencies in the calcium alginate bead (Redenbaugh et al., 1991). Similar result also observed by Rai et al. (2008) in conversion frequency of encapsulated shoot tips of *Psidium guajava*. However, this result was contradict from conversion frequency of synthetic seeds of mulberry (Patnaik et al., 1995) strawberry and raspberry (Lisek & Orlikowska, 2004), low temperature (4.0 ºC) was found to be a good storage temperature.

The results of the present study clearly showed that the low temperature storage (4.0 ± 1 ºC) was less appropriate technique for synthetic seed of *Dioscorea alata*. Therefore, further experiments for the development of the better technique is required. For example, the used of *in vitro* storage under minimal growth condition. Through this technique, slow growth was induced by halving of the nutrient supply like synthetic seeds were cultured on sucrose lacking medium or removal of exogenous plant plant growth regulators. Several researchers have successfully demonstrated the use of minimal growth media for slow growth treatments for the conservation of germplasm. *In vitro* storage through minimal growth is now routinely used for conversion of several plant species like banana, potato and cassava (Ashmore, 1997).

Tissue culture was considered successful when plantlets obtained were successfully acclimatized to the natural environment (*ex vitro*). Thus, in Chapter 4 section 4.5, acclimatization of *Dioscorea alata* plantlets to the greenhouse was carried out. Growth substrates play as a crucial factor in acclimatization process. Substrates with greater nutrient levels would promote *Dioscorea alata* growth in terms of shoot, root and tuber vigorously. In order to establish the optimum growth of plantlets transferred, four substrates, which were Black soil, Red soil, Vermicompost and Culite were analyzed to determine their nutrient contents. The suitable substrate as sowing medium for *Dioscorea alata* acclimatization were decided from the soil content analysis.

The data on chemical and fertility properties of the substrates tested were presented in Table 4.24. The pH of a soil is among the most important soil characteristics for crop production. The pH of a soil is a measure of the activity of hydrogen (H+) ions in the soil solution usually obtained by shaking soil with distilled water. The pH of the soil influences plant growth by controlling the nutrient availability. The optimum pH required for *Dioscorea sp*. growth are from 6.0 to 8.0. Based on results obtained in Table 7.1, the

pH of four types of sowing media were between 7.0 to 8.0 which was suitable for *Dioscorea alata* growth. *Dioscorea sp.* tolerates fairly wide variation in soil pH, though very acid soils should be avoided. Lower pH can cause toxic to plants by damaging the root tips and interfere the uptake of essential nutrient such as calcium. As soils become increasingly acidic, important nutrients like phosphorus become less available to plants. Other elements, like aluminum, become more available and may actually become toxic to the plant, resulting in reduced crop yields.

The exchangeable cation is defined as interchange between a cation on the surface of negatively charged particles such as humus and fine clay mineral in the soil. For acidic soil solution, concentration of H^+ and Al^{3+} are high and this ions dominate the absorption of cations (absorption of H^+). For natural and alkaline soil solution, both H^+ and Al^{3+} are low and Ca^{2+} and Mg^{2+} are higher cause lower the absorption of H^+. This indicates that the amount of basic cation that occupies the cation exchange site divided by Cation exchange capacity (CEC). The increase of pH value is dependent on the saturation of base.

Cation exchange capacity (CEC) is the capacity of the substrate to hold cations in soil particles surfaces. CEC was calculated from the analysis results of potassium (% K), calcium (% Ca), magnesium (% Mg), sodium (% Na) and Buffer pH (% H). CEC was effected by the soil clay and organic matter content, so CEC can be considered as a generalized indicator of the soil texture. The value of CEC was different among the four types of sowing media. Black soil and Red soil typical texture were loamy soils with CEC 15.57 meq/100g and 15.03 meq/100 g, respectively. Culite typical texture was clay soil with CEC 33.55 meq/100g. High CEC, 88.77 meq/100 g was observed from Vermicompost. Generally, extremely heavy clay soil was not recommended as sowing media for *Dioscorea sp.* as they restricted the tubers growth and harvested. It is reported

that yam is well grown and highly harvested for tubers in sandy loam or loamy soils than sandy or clayey soils (Cho et al., 1995; Park et al., 2000).

Potassium (K) is one of the most abundant elements in soil. K is an essential element that associate with water movement, nutrient and carbohydrate in plant tissues. K has several important functions in the plant growth, such as stimulates early growth of vegetative and increase the protein production. K also can improve the efficiency of water use and improve the level of resistance to diseases and insect attacked. Based on Table 4.24, Black soil contained high potassium with 0.85 meq/100g. Calcium (Ca) is one of the elements that is constituent of the cell wall and apparently influences its elasticity. It also involved in cell division in meristematic tissue and play important role in enzymatic and hormonal process. Culite contained the highest Ca (12.27 meq/100g) and Black soil possessed the lowest Ca (0.32 meq/100g). Magnesium (Mg) is an important element constituent of chlorophyll and hence play important role in photosynthesis process. Red soil was detected with the lowest Mg content (0.28 meq/100g). Sodium (Na) may indicate potential for soil structure breakdown and water percolation problem. Black soil (0.11 meq/100g), Red soil (0.06 meq/100g) and Culite (0.34 meq/100g) showed very low sodium hazard. Vermicompost with high Na (6.21 meq/100g) was low sodium hazard.

Percentage of Organic carbon is the measure of the percentage of total carbon-containing materials in a substrate. The organic matter content may affect certain herbicide application rates and may affect fertilizer rate recommendations for nitrogen or sulfur. Vermicompost contained the highest percentage of organic carbon 21.19 %. Red soil contained the lowest percentage of organic carbon 0.36 %. Relatively high organic matter content of these sowing media allows for a high rate of nutrient release to the plant, and optimum water retention capacity. According to O'Sulllivan & Ernest (2008) *Dioscorea sp*. require a high level of natural soil fertility. Since soil organic matter plays

a fundamental role in yam productivity, which is not compensated in a soil with low organic content by the addition of inorganic fertilizers, all management practices that will favour the maintenance or buildup of soil organic content must be evaluated in yam production systems (Diby et al., 2009).

Total Nitrogen measures the total amount of N present in the soil, much of which is held in organic matter and not immediately available to plants. Nitrogen (N) is known to be one of the most important single factors limiting the production of the *Dioscorea* species tubers (Aduayi & Okpon, 1980) but, recently, Diby et al. (2009) reported that potassium (K) may be more limiting to yam growth than N. Black soil, Red soil and Culite contained very low total N. Vermicompost showed high total N. Kayode (1985) reported that N at 35 kg ha-1 was optimum for successful production of *Dioscorea alata* that had been cultivated for two years. However, Amon & Adetunji (1970) recommended N rates of 25 to 56 kg ha-1. In potatoes, for example, a high level of nitrogen fertilization results in stronger development of shoots and a reduction of tuber yield due to a delay in tuber bulking and a smaller harvest index (Biemond & Vos, 1992), while on the other hand, K application improves the transfer of assimilates to the tubers (Marschner, 1995). For yams, Enyi (1972) observed that K application increased *Dioscorea esculenta* yield by increasing tuber bulking rate as well as bulking duration phase through induction of earlier tuber initiation.

Most soil tests will measure the readily available Phosphorous (Available P) and some component of the phosphorous (organic/inorganic) which becomes available or may be available (Total P) when available P was depleted by plant uptake. From the results in Table 7.1, Black soil showed extremely low Available P (7.0) but high Total P (388). Red soil contained moderate Available P (150.0) and low Total P (107.0). Extremely low Available P (4.0) and moderate Total P (209.0) was detected in Vermicompost. In culite,

the Available P was moderate (197.0) and Total P was high (391.0). However, Phosphorus was not considered to be limiting as *Dioscorea* species have low requirements in P (O'Sullivan & Jenner, 2006; O'Sullivan & Ernest, 2008; Vander Zaag et al., 1980).

Trace elements occur naturally in soils, some are essential micronutrients for plants and animals and are thus important for human health and food production. Trace elements were needed in small quantities by plants. At elevated levels all trace elements however, become potentially toxic. Although only required in small amounts, trace elements are essential for plant growth. Critical levels for trace elements will vary between soil types and plants. From the results in Table 4.24, all four types of sowing media tested showed the presence of trace elements such as Fe, Mn, Cu and Zn. Fe was needed in plant as a constituent of many compounds that regulated and promoted growth. Mn was the micronutrient that helping plant in photosynthesis. Cu is an essential constituent of enzymes in plants and Zn helps in the production of a plant growth regulators responsible for stem elongation and leaf expansion.

Dioscorea species requires high level of nutrient for growth (O'Sullivan & Ernest, 2008). Nitrogen (N) and potassium (K) are largely stored by the tubers (Diby, 2005; O'Sullivan & Ernest, 2008) while the calcium (Ca) is mainly accumulated in the leaves and returns to the soil with dead leaves (Diby et al., 2009). A major constraint for enhancing yam productivity is low soil fertility, both in terms of macro and micronutrient deficiency (Osullivan & Ernest, 2007). This is because yams are high-nutrient-demanding species (Carsky, 2007), and when planted in low-fertility soils under subsistence yields are low (Diby et al., 2009). Therefore, the selection of a suitable substrate can be decisive for acclimatization. Based on observations from soil chemical analysis, physical analysis

and soil texture, Black soil and Red soil was selected as the suitable sowing media for *Diascorea alata* plantlet acclimatization.

In the present study, micropropagated plantlets with well-developed roots (Figure 4.22) were transplanted to Black soil, Red soil and a combination of Black and Red soil at a ratio of 2 to 1 for plantlets acclimatization. Direct transfer of plantlets to *ex vitro* conditions, micropropagated plants are very susceptible to various stresses because they have not yet developed adequate patterns of resource allocation and morphological and physiological features required by the new environment (Chaves, 1994). Low photosynthesis rates (Grout & Aston, 1978; Cournac, et al., 1991) and the malfunctioning of the water housekeeping system (Capellades et al., 1990) are the two of the major constrains in tissue cultured plants. When plantlets are in culture, it only used a fraction of CO_2 because it is constantly supplied with a carbon energy source, however when exposed to an *in vivo* environment it becomes autotrophic (Hoe, 1992). Plantlets are stressed because of the stomatal development are not complete. Besides suffer light stress (photoinhibition), plantlets also suffer water stress due to the differences between *in vitro* and *ex vitro* relative humidity. In *in vitro* environment the culture was supplied with high level of humidity but when plantlets were transferred to the greenhouse, the level of humidity was decreased because of direct light intensity.

Therefore, in this study, gradual decrease of relative humidity was done by keeping the plantlets in culture rooms at 25 ± 1 °C under 16 hours light and 8 hours dark for 4 weeks, watered every day, covering the plantlets with transparent plastics with small holes and the plastic covered was stepwise opened (Figure 4.23). Razdan (1993) suggested by covering acclimatized plantlets with clean transparent plastic bags having small holes for air circulation, high humidity can be built up around transplanted plants. By this acclimatization process the *in vitro* plantlets features will slowly develop to the

level of intact plant. Kranz (1996) and Remigio et al. (2003) stated that high humidity often causes shoot elongation and increase fresh weight and leaf area. According to Ziv et al. (1992), the low relative humidity condition of the culture vessels is important in order to develop more vigorous plantlets structure and therefore, plantlets could adapt easily when transferred to the field.

The percentage of survival rate varied with type of substrates. The data recorded on the survival rates of the plantlets during acclimatization was presented in Table 4.25. A significant percentage of 93.33 ± 0.05 % was obtained with mixture of Black soil and Red soil while 83.33 ± 0.09 % of survival rate was observed from plantlets acclimatized in Black soil. The lowest survival rate (70.00 ± 0.09 %) was observed from plantlets acclimatized in Red soil. The highest percentage of survival rate was obtained from a mixture of black soil and red soil compared to only Black soil and only Red soil. This results might be due to the differences in soil properties. According to soil analysis, Red soil was a little bit sandy and typically indicates weathering and good drainage and the data clearly presented the greater fertility of the Black soils so a mixture of Black and Red soil might provide enough nutrients, good drainage and aeration system for the plantlets growth. Similar effect were observed in acclimatization of *Dioscorea sp.* (Behera, et al., 2010; Fotso et al., 2013; Bazakana et al., 1999), a mixture of sandy soil produced better percentage of acclimatization. Da Silva et al. (2005) demonstrated that the greater the aeration the more efficient the survival and the higher the growth and vigour of the plant. Sink (1984) obtained similar results and he stated that retain water will deplete oxygen level in the soil and thus, this causes failure of the plant rooting system. Debergh et al. (1990) also stated that the soil should not be too wet and the exposure to higher light intensity should be gradual. The lower soil organic matter, clay and exchangeable bases explained the poorer growth of acclimatized *Dioscorea alata* in Red soil.

The percentage of survival rates of plantlets were declined in all treatments after 8 weeks being acclimatized. This might be due to poor adaptation to high environmental temperature. However, only small percentage of decreasing was detected. As presented in Table 4.25, in the mixture of Black soil and Red soil, a decline of 3.0 % was observed with the current (8 weeks) percentage of 90.00 ± 0.06 %. It was still considered as great percentage of survival rate of *Discorea sp.* acclimatization. Das et al. (2013) managed to acquire only 85 to 87 % of survival rates of *Dioscorea alata* after 8 weeks of hardening. Behera et al. (2010) successfully obtained the same result with current study that was 90% survival rate after 4 weeks of being transferred. Black soil and Red soil showed percentage of acclimatization 73.33 ± 0.08 % and 56.67 ± 0.09 %, respectively.

A wide range of plant macromorphology such as plant height, number of leaves and number of node were observed from acclimatized plantlets compared to *in vitro* plantlets. The micropropagated plantlets grew normally without any morphological variation between the *in vivo*, *in vitro* and acclimatized plantlets. Table 4.26, presented the observations on macromorphological characteristics of *in vitro* grown and acclimatized plantlets of *Dioscorea alata*. Upon the addition of growth substrates, plantlets started to produce new shoots and roots. The plants grew well and attained 8-10 cm in height within 4 weeks of transfer (Figure 4.24) and 15 to 16 cm after 8 weeks of transfer (Figure 4.25). The stem was herbaceous vine with internodes square in cross section, cylindrical and purple in colour. Stem started to twine along the stand prepared after 8 weeks of transfer. The same characteristic of stem was observed from intact or *in vivo* grown plant (Table 4.1). Maintained the ovule, narrowly heart shape and colour, the size of leaves became bigger and the number of leaves also increased after 8 weeks of acclimatization. From the observation average number of nodes was the same as average number of leaves produced by plantlet. After 8 months of acclimatization, plants started to produce tubers.

Tubers of *Dioscorea alata* have vivid purple colour which are verified as anthocyanin pigment. Anthocyanins are natural, water soluble, nontoxic pigments that responsible for blue, red and purple colours of the fruits, vegetables, flowers, roots, tubers and other plant tissues. At the beginning, anthocyanin pigment was used for fabric dyes industry. More recently, with the advent of prepared and processed foods, the pigment have become increasingly popular as natural food colourant (Bridle & Timberlake, 1996) due to their attractive colours. Nowadays, there is considerable demand for the use of anthocyanin as natural colourant due to the increasing consumer preference for healthy foods and concern about the safety of synthetic colourant. Existing evidence indicates that the anthocyanin are not only non-toxic and non-mutagenic, but have positive therapeutic properties (Saija, 1994). *Dioscorea alata* is a relevant source of natural pigment.

In addition to their ability to provide vibrant purple colour, they are widely acknowledged as having significant health-giving properties such as an antioxidant, anti-inflammatory, anti-ulcer and wound healing properties. Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species (ROS) such as singlet oxygen, superoxide, peroxyl radicals, and peroxy nitrite which result in oxidative stress leading to cellular damage. Oxidative metabolism is essential for the survival of cell. A side effect of this dependence is the production of free radicals and other reactive oxygen species that cause oxidative stress. However, oxidative stress initiated by free radicals that seek stability through electron pairing with biological molecules in healthy cells and cause protein and DNA damage along with lipid peroxidation often leads to irreversible imbalance in cellular organization which is a major problem of concern. These oxidative changes are implicated in the pathogenesis of various human diseases such as myocardial, cerebral ischemia, arteriosclerosis, diabetes, rheumatoid arthritis, inflammation, cancer initiation and aging process.

However, there are several limitations to the current supply of anthocyanin pigment from *Dioscorea alata* tubers. The factors that restrict rapid conventional propagation and economic exploitation of *Dioscorea* species include the absence of viable seeds, long period for obtaining usable tubers and phytosanitary problems (Bolagum et al., 2009; Tschannen et al., 2005). Tubers yield also drastically reduced by viral, fungal and nemathods infection. All of these factors affect the quality and quantity of tubers hence anthocyanin production. Therefore, the present study also focused on anthocyanin content from *in vitro* samples (callus and plantlets). Chapter 4 section 4.6 discussed experiments on anthocyanin production and accumulation of *Dioscorea alata* L. in *in vivo* and *in vitro* grown plants.

The total anthocyanin content was estimated according to the spectrophotometric pH differential method by Gabriela et al. (2010) and Lee et al. (2005). The method can be used for the determination of total monomeric anthocyanin content based on structural change of the anthocyanin choromophore between pH 1 and pH 4.5. The concept of this method is determining the amount of anthocyanin present in a sample by measuring the change in absorbance at two different pH. This method has been used extensively by food technologist and horticulturist to assess the quality of fresh and processed fruits and vegetables (Lee et al., 2005). Wrolstad et al. (1995) stated that, the pH differential method has been described as fast and easy for quantitation of total monomeric anthocyanin.

To calculate the total anthocyanin content, samples of *Dioscorea alata* were prepared at pH 1 by diluting the samples with 0.025 M potassium chloride buffer and pH 4.5 using 0.4 M sodium acetate buffer. At pH 1, the extract from purple colour *Dioscorea alata* tuber become red and clear at pH 4.5 (Figure 4.26). The colour changes upon exposure to different pH, might be due to the change in the pigment's structure. There are four major anthocyanin forms that exist in equilibrium; the red flavylium cation, blue quinonoidal

base, colourless carbinol pseudobase and colourless chalcone. According to Kohno et al. (2009) increasing of pH values could cause red flavylium cation to deprotonate easily to form the voilet quinonoidal base. Brouillard (1988) observed that as the pH is raised, kinetic and thermodynamic competition occurs between the hydration reaction on position two of the flavylium cation and the proton transfer reaction related to its acidic hydroxyl group. This shows that anthocyanin undergoing several reactions as the pigment is destroyed which affect the colour.

Spectrophotometric measurement carried out (Figure 4.27) clearly showed that the wavelength of maximum absorption for anthocyanin of *Dioscorea alata* was at 530 nm with absorbance value of 0.3802 in pH 1.0 potassium chloride buffer. Unlike pH 4.5, the curvature of pH 1.0 was more obvious in the visible region between 460 to 560 nm. There was almost no peak of absorbance for pH 4.5 as the absorption band increase very slightly between 530 to 550 nm. The pH differential method is based on the assumption that monomeric anthocyanin have little or no absorbance in pH 4.5 buffer and that polymeric or degraded anthocyanin will be absorbed at this pH. Although nearly all monomeric anthocyanins are in hemiketal form at pH 4.5, a small proportion are in the quinoidal form or the flavylium form, which will make a small contribution to the absorbance in pH 4.5 buffer (Lee et al., 2005).

Comparative study of anthocyanin content in *in vivo* and *in vitro* samples of *Dioscorea alata* was also carried out in the present study. Another interesting fact about tissue culture, its offers an opportunity to exploit the cell, tissue, organ or entire organism by growing them *in vitro* to get desired compounds. This is because plant cells are biosynthetically totipotent, which means that each cell in culture retains complete genetic information and hence is able to produce the range of chemicals found in the parent plant (Ramachandra & Ravishankar, 2002).

The total monomeric anthocyanin content from *in vivo* (leaves and tubers) and *in vitro* (callus and plantlets) samples of *Dioscorea alata* were provided in Table 4.27. The total monomeric anthocyanin content was calculated as cyaniding-3-glucoside. Cyaniding-3-glucoside was chosen because it is the most common anthocyanin in nature (Francis, 1989) and several investigators have determined its extinction coefficient with values ranging from 18800 to 34300 L x mol^{-1} x cm^{-1} depending on the solvent and wavelength of the maximum absorbance. Absorbance of the mixture was measured at 530nm ($\lambda_{vis-max}$ of the pigment solution) and 700nm using a UV-Vis spectrophotometer.

The anthocyanin content in tubers was 341.99 ± 0.18 mg cya-3-glu / 100 g FW and leaves 247.92 ± 0.71 mg cya-3-glu / 100 g FW whilst in callus 295.21 ± 0.20 mg cya-3-glu / 100 g FW and plantlets had a value of 175.25 ± 0.42 mg cya-3-glu / 100 g FW. The total monomeric anthocyanin content in tubers was higher than in leaves and from *in vitro* sample. For *in vitro* samples, callus and plantlets were initiated from shoot tip explants (develop into leaves). According to Matkowski (2008), in dedifferentiated cells, some biosynthetic potential typical for the developed organs from they were initiated can be conserved. This statement supported the current result, anthocyanin content in *in vitro* sample was lower than tuber sample. Matkowski (2004) also found that in *Pueraria lobata* callus culture, the bioactive isoflavonoid content dependent on source organs. Therefore selection of the starting explants was very important in anthocyanin accumulation in *in vitro* culture.

However, the total monomeric anthocyanin in callus (*in vitro* sample) was higher than in leaves (*in vivo* sample). This result was supported by fact that the growth and development of plants is modulated by interaction between many endogenous factors, including plant growth and changes in the endogenous concentration of the PGRs and the tissue sensitivity in the various plant organs control a wide range of developmental

processes. The synthesis of anthocyanins is also suggested to be under hormonal control (Weiss et al., 1995; Peng et al., 2011; Nagira et al., 2006). In this recent study of anthocyanin content, culture media for *in vitro* regeneration was supplemented with BAP and 2,4-D. Fang et al. (1998), reported that BAP supplementation to the suspension culture of *Vaccinium palahae* in a concentration of 20 µM increased three times anthocyanin synthesis, and accelerated their production. Piazza et al. (2002) found that 25 µM BAP enhanced the response to light in *Zea mays*, but was not sufficient to induce anthocyanin accumulation in darkness. Anthocyanin synthesis can also be enhanced by exogenous 2,4-D. Bae et al. (2012) reported that 1.0 mg/l of 2.4-D resulted in the highest production of anthocyanin (26 times higher), as compared to the control transgenic root cultures of *Raphanus sativus*. Nakamura et al. (1998) stated that, low concentration of 2.4-D in the medium enhanced both anthocyanin production and anthocyanin methylation in strawberry suspension culture.

For *in vitro* samples, anthocyanin content in callus was higher than those found in regenerated plantlets. According to Matkowski (2008), undifferentiated cell (callus) are able to accumulate great amounts of phenolic acid. This result was in contrast with the finding if Kintzios et al. (2004), whereby a lower accumulation of rosmarinic acid of *O. basilicum* cell culture compared to in regenerated plantlets.

Although the capability for the formation of anthocyanin is generally determined by hereditary factors, the amount of pigment produced is affected by numerous environmental factors such as nutrition and water availability, age of stock plant, temperature and light (Mancinelli et al., 1983; Rabino, 1977). Light influenced the synthesis of anthocyanin by regulating the level and activity of biosynthetic enzymes (Werner et al., 1998; John & Wesley, 1991). Light has been utilized to improve the efficiency of tissue culture system for many plants but there are insufficient researches

focused on the effect of light/dark cycle on callus formation and anthocyanin production. Determination of the sensitivity of explants produce callus towards light may result in better utilization of light as a regulator in coloured callus production.

To compare the effects of light on anthocyanin content, light/dark cycle (photoperiods) were manipulated on coloured callus formation and anthocyanin production. Four different colours of callus (dark purple, reddish purple, purple, purplish white) were observed to form after 2 weeks incubation of callus cultures which maintained in the culture room at different photoperiods (24L/0D, 16L/8D, 12L/12D and 0L/24 D), respectively. Pigments selectively absorb and reflect specific wavelengths of light, thus giving the pigment its distinctive colour. After 8 weeks of culture, the purple pigment become more visible and anthocyanin accumulation were comparable among the treatments. The coloured callus extracted with 97% ethanol were diluted in pH 1.0 and pH 4.5 buffers (Figure 4.28) before subjected to UV-VIS spectrophotometry. At pH 1.0, the purple colour of extract solution became red and at pH 4.5, the purple colour became clear. According to Lee et al. (2005), monomeric anthocyanin undergo a reversible structural transformation as a function of pH, coloured oxinium form at pH 1.0 and colourless hemiketal form at pH 4.5.

Figure 4.29 showed spectral characteristic of crude anthocyanin extracts. In the visible region between wavelength 450 to 570 nm, the curvature of anthocyanin extract in pH 1.0 was more obvious compared to in pH 4.5. There was almost no absorption peak for pH 4.5 as the absorbance reading slightly increased between 530 to 550 nm. Spectrophotometric analysis clearly showed that the maximum absorption for the anthocyanin was 0.3802 at 530 nm in pH 1.0. Based on Figure 4.29, total monomeric anthocyanin was calculated using spectrophotometric pH differential method.

Table 4.28 presented anthocyanin content of *Dioscorea alata* coloured callus which exposed to different photoperiods. The result showed callus exposed to different photoperiods produced different amount of anthocyanin content. The highest anthocyanin content was 295.21 ± 0.20 mg cya-3-glu / 100 g FW detected in coloured callus maintained at 16L/8D photoperiod. The lowest anthocyanin content, 90.35 ± 0.32 mg cya-3-glu / 100 g FW was observed from callus exposed to dark environment. These results indicated that light have influenced the level anthocyanin production. Light has been found to affect the synthesis of anthocyanin by influencing the level and activity of biosynthetic enzymes (Werner et al., 1998; John & Wesley, 1991). The biosynthesis of anthocyanin is controlled by genes, such as phenylalanine ammonialyase (PAL), chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), anthocyanidine synthase (ANS), dyhydroflavono-4-reductase (DFR) and UDP-glycose flavonoid glycosil transferase (UFGT). Phenylalanine ammonia lyase (PAL) and the enzymes that convert cinnamate to p-coumaroyl-CoA are rapidly induced with light, hence promoting the synthesis of anthocyanins (Giovanni et al., 2011).

In *Perilla frutescens* cell suspension culture, light had been observed to be a positive regulator for anthocyanin biosynthesis with a 1.6 g/l anthocyanin yield was obtained (Zhong et al., 1993). Anthocyanin accumulation was enhanced by 13.2 fold reported when maintained under continuous light irradiation in methyl jasmonate elicited *Vitis vinifera* (Zhang et al., 2003). Blando et al. (2005) reported, in cultured cherry *Prunus cerasus* callus and suspension, the light exposure caused the rapid change of the tissue colour from white to purple. This observation was in agreement with the current study, whereby whitish coloured callus was obtained from explants cultured and maintained in the dark.

In the present study, light was the crucial factor that affect the accumulation of anthocyanin in callus. From the results in Table 4.28, the longer the light exposure the more amount of anthocyanin was detected. However, the amount of anthocyanin content was lower, 245.46 ± 0.13 mg cya-3-glu / 100 g FW from callus exposed at photoperiod 24L/0D compared to callus exposed to photoperiod of 16L/8D. This result suggested that other than 24 hours light supply, light/dark cycle or photoperiod was important in anthocyanin accumulation in callus culture of *Dioscorea alata*. Neyland et al. (1963) supported the current result by stated that leaves and stems of *Kalanchoe blossfeldiana* accumulated significant amounts of anthocyanins as a consequence of photo-periodic response. How & Smith (2003) also found similar result where the highest yield of anthocyanin in *Ajuga reptens* callus obtained on 16L/8D treatment.

Other than anthocyanin accumulation, the recent study also focused on production of phenolic acid and flavonoids in *in vitro* culture. Phenolic compounds, widely existing in plants are important for their contribution to colour, sensory attributes and food nutrition. Anthocyanin represent a group of water soluble phenolic compound responsible for the blue, purple and red of many fruits and vegetables. Phenolic acids and flavonoids are 2 major polyphenolic compounds that can be attributed to the antioxidant capacity in plants. Phenolic compounds are reported to have multiple biological effects including antioxidant activity, antitumor, antimutagenic and antibacterial properties. Kirakosyan et al. (2003) reported that phenolic compounds in plant possess antioxidant activity and may help protect cells against the oxidative damage caused by free radical.

In the present study, total phenolic and flavonoid content from *in vitro* sample (callus) was calculated and compared with total phenolic and flavonoid found in *in vivo* sample (tuber). Different bioactive compounds were detected in *in vitro* cultures especially callus culture such as flavonoids (Maneechai et al., 2012), cardenolides (Sahin et al., 2013) and

phenolic acids (Szopa et al., 2014). Numerous alkaloids, saponins, cardenolides, anthraquinones, polyphenols and terpenes have been reported from *in vitro* cultures and reviewed several times (Misawa, 1994; Verpoorte et al., 2002; Vanisree & Tsay, 2004).

As shown in Table 4.29, in *vivo* and *in vitro* samples of *Dioscorea alata* contained both phenolic compounds and flavonoids. The total phenolic content of *in vivo* sample was 73 mg 100 g^{-1} and *in vitro* sample was 52 mg 100 g^{-1}. While total flavonoids of *in vivo* sample was 48 mg 100 g^{-1} and *in vitro* sample was 21mg 100 g^{-1}. As shown in Table 4.29, the concentration of total phenolic and flavonoid content were significantly higher in *in vivo* samples compared to *in vitro* samples. These could partly be due to the differences in samples used, maturity and other growing conditions. Callus extracts were used as *in vitro* sample. According to Ramachandra & Ravishankar (2002) production of secondary metabolites in plants generally higher in differentiated tissues like shoots and roots, since these organ cultures are relatively more stable.

Recent interest in these compounds had been stimulated by the potential health benefits arising from their antioxidant activities and free radical scavenging capacities in coronary heart disease and cancer (Yao et al., 2004). Phenol antioxidant index is a combine measure of the quality and quantity of antioxidant and is responsible for effective free radical scavenging and antioxidative action. The antioxidant capacity of phenolic compounds has long been accepted for their ability to scavenge radicals and strong chain-breaking actions, thereby protecting cells against the detrimental effects of reactive oxygen species (Routray et al., 2013). Flavonoids are recognized for their high pharmacological activities as radical scavengers. Flavonoids are important secondary metabolites of plant modulating lipid peroxidation involved in antherogenesis, thrombosis, carcinogenesis, hepatotoxicity and a variety of disease conditions (Sakthidevi & Mohan, 2013).

Phenolic compounds and flavonoids are well known example of secondary metabolites with antimicrobial activity produced by plants. There are reports showing that alkaloids and flavonoids are responsible for the antifungal activities in higher plants (Cordell et al., 2001). Besides, secondary metabolites such as tannins and other compounds of phenolic nature are also classified as active antimicrobial compounds. Nowadays, production of secondary metabolites and valuable compounds as well as antimicrobial agent from *in vitro* is preferred as an alternative to prevent devastation and extinction of plant species besides offers the enhancement of desirable compounds through modification of medium for regeneration. Interestingly, the current investigation of *Dioscorea alata* revealed that extracts from both *in vivo* and *in vitro* samples possess phenolic compound sand flavonoids. Therefore, current study (Chapter 4 section 4.7) of *Dioscorea alata* focused on evaluation of antimicrobial (antibacterial and antifungal) activity from *in vivo* (tubers) and *in vitro* (plantlet and callus) samples of *Dioscorea alata* L. using disk diffusion method.

Disk diffusion method was used widely to investigate the antimicrobial activity of natural substances of plant extracts. Disk diffusion method offers many advantages like simplicity, low cost, the ability to test enormous number of microorganism, antimicrobial agent and the best to interpret results provided (Baloury et al., 2016). This method developed in 1940 (Heatley, 1944), was the official method used in many clinical microbiology susceptibility testing. Although not all fastidious bacteria can be tested accurately by this method, the standardization has been made to test certain fastidious bacteria pathogens like streptococci, *H. influ*enze, *N. gonorrhoeae* and *N. meningitides* (CISI, 2012). In this procedure, agar plates were inoculated with a standardized inoculum of the test microorganism. Then, filter paper discs (6 mm), containing the test compound at a desired concentration were placed on the agar surface. Generally, antimicrobial

agents diffused into the agar and inhibits germination and growth of the test microorganism.

Chloramphenicol and carbendazim were selected as the positive control for antibacterial and antifungal studies. Chloramphenicol is a broad-spectrum antibiotic that inhibit mitochondrial protein synthesis by hinders protein chain elongation by peptidyl transferase inhibition of bacterial ribosome. Chloramphenicol is active against a variety of organisms. Chloramphenicol was discovered after being isolated from *Streptomyces venezualae* in 1947 (Pongs, 1979). Chlorampheenicol is the most effective and medicines needed in a health system according to WHO list of essential medicines. Carbendazim is reported to interfere with mitosis and cell division in fungi. Carbendazim inhibits the aggregation of the spindle microtubules and disturbed the alignment of chromosoms in the central plate of fungal methaphase. Carbendazim, methyl 2-benzimidazole carbamate, is a systemic benzimidazole fungicide and used to control a broad range of diseases on arable crops, fruits, vegetables, ornamentals and medicinal herbs. It is also a main metabolic product of some other systemic fungicides, such as benomyl and thiophanatemethyl (Fleeker et al., 1974; Mongomery, 1997).

Extraction efficiency is affected by the chemical nature of phytochemicals, the extraction method used, sample particle size, the solvent used as well as the presence of interfering substances (Stalikas, 2007). The yield of the extraction depends on the solvent with varying polarity, pH, temperature, extraction time and composition of samples are known as the most important parameter (Do et al., 2014). In the present study, *Dioscorea alata* extracts were obtained by using water and ethanol. The common used reagent for isoflavone (antimicrobial) extraction was methanol. Although methanol was considered as a good solvent extraction, however it was toxic. Therefore, in this study for antimicrobial activity, ethanol and water were used as the extraction solvents. Ethanol has

been known as a good solvent for polyphenol extraction and is safe for human consumptions. According to Sartory & Grobbelaar (1984), ethanol was the best solvent extraction among the others which were used to extract pigments from freshwater phytoplankton. For extracting flavonoids from tea, aqueous ethanol performed better than aqueous methanol (Wang & Helliwell, 2001).

Two gram-positive (*S. aureus* and *S. epidermis*) and two gram-negative (*E. coli* and *Salmonella sp.*) bacteria were used to screen antibacterial activity in *Dioscorea alata*. *S. aureus*, a phyrogenic bacterium is one of the most common gram-positive bacteria causing food poisoning, well known to play a significant role in invasive skin diseases including superficial and deep follicular lesions. *S. aureus* was resistant to antibiotic methicillin. *S. epidermidis*, is the same genus with *S. aureus*. Several studies have identified *S. epidermidis* as a common colonizer of healthy human skin (Gao et al., 2007; Kloos & Musselwhite, 1975) and mouse skin (Grice et al., 2008). *S. epidermidis* strains are often resistant to antibiotics including methicillin, gentamycin and tetracyclin. *E. coli* is a gram negative bacteria, belonging to the normal flora and humans. However, an enterohemmoragic strain of *E. coli* has caused serious cases of food poisoning and preservative to eliminate its growth are needed. *E. coli* may also be the causative organism in appendicular abscess, peritonitis, cholecystitis and wound infections (Hugo, 1994). *Salmonella sp*. is known to infect a number of human and animal species.

The present study showed that ethanolic extract of *Dioscorea alata* exerts antibacterial activities. Negative control was run in parallel to ensure the inhibition could be properly attributed to the extract rather than ethanol solvent. In the present study, ethanol solvent showed antibacterial effect against *S. aureus*, *S. epidermis*, *E. coli* and *Salmonella sp*. Inhibition zones were observed. Although it is possible that the ethanol solvent was extracting antimicrobial compounds that are accounting for the large zone of inhibition

observed but it was not significant. *Dioscorea alata* sample extracts exhibited larger inhibition zone compared to ethanol solvent. However, the used of 95% ethanol must be cautioned due to the antibacterial activity of the control.

The growth inhibitory activities of ethanolic extracts of *in vivo* and *in vitro* samples of *Dioscorea alata* are summarized in Table 4.30. At the concentration of 20 µL both *in vivo* and *in vitro* samples showed higher diameter of inhibition zones compared to negative control. The ethanolic extract of *in vivo* (tubers) samples exhibited antimicrobial activity against *S. aureus*, *S. epidermis*, *E. coli* and *Salmonella sp.* showing zone of inhibition of 18.33 ± 0.88 mm, 23.00 ± 1.73 mm, 12.00 ± 0.58 mm and 18.00 ± 1.00 mm respectively. Ethanolic extract of *in vitro* samples (plantlets) showed inhibition zones against *S. aureus* (10.00 ± 0.58 mm), *S. epidermis* (12.00 ± 1.52 mm), *E. coli* (12.33 ± 0.67 mm) and *Salmonella sp.* (10.33 ± 0.33 mm). Ethanolic extract of *in vitro* (callus) exhibited activity against *S. aureus*, *S. epidermis*, *E. coli* (Figure 4.30) and *Salmonella sp.* (Figure 4.31) with inhibition zone of 9.33 ± 0.67 mm, 10.67 ± 1.14 mm, 7.00 ± 0.58 mm and 10.00 ± 1.00 mm, respectively.

These results illustrated that the antibacterial activity and susceptibility test obtained in this study varied according to the parts of the plants used. Among the samples tested, tubers were very promising and showed significant inhibition against all tested bacterial. The growth of *S. aureus*, *S. epidermis E.coli* and *Salmonella sp*. were strongly inhibited by tuber extracts. Similar result was observed in *Dioscorea deltoidea*, tubers ethanolic and water extract have antibacterial effect against *S. aureus*, *S. epidermis*, *E .coli* and *Salmonella sp*. (Chandra et al., 2013). Other studies of *Dioscorea sp*. also showed significant antibacterial activity from tuber extracts. . Extracts from tubers of *Dioscorea hirtiflora* and *Dioscorea dumetorum* have bacterial effect against *S. aureus* and *E.coli (*Sonibare & Abegunde, 2012). Ethanolic extracts of *Dioscorea pentaphylla* tubers

showed remarkable antibacterial activities against *S. aureus* (Prakash & Hosetti, 2010). *Dioscorea bulbifera* tubers extract showed growth inhibition against *E.coli* (Kuete et al., 2012). Plantlets and callus showed significant higher antibacterial activity compared to negative control.

In vivo and in *vitro* extracts showed moderate inhibition activity against *E.coli*. In general, the extract of plants are more effective against Gram-positive than Gram-negative bacteria. According to Kumar et al. (2006), antibiotic substances appear to be more inhibitory to Gram-positive bacteria than to Gram-negative type. This tendency may be due to several possible reasons such as permeability barrier provided by presence of cell-wall with multilayer structure in Gram-negative bacteria or the membrane accumulate in mechanisms or presence of enzymes in periplasmic space which are able to breakdown foreign molecules induced from outside (Holetz et al., 2002; Abu-Shanab et al., 2004; Parekh & Chanda, 2007). The lipopolysaccharide layer along with the protein and phospholipids are the major components in the outer surfaces of Gram-negative bacteria (Burn, 1988). The lipopolysaccharide layer can hinder the access of most compounds to the peptidoglycan layer of the cell wall. This fact explains the resistance of Gram-negative bacteria to the lytic action of most extracts exhibiting activity. This present result therefore supports the fact the Gram-negative bacteria are more resistant than the Gram-positive bacteria. Several previous studies (Branter et al., 1996; Nostro et al., 2000; Ojala et al., 2000) reported that Gram-negative bacteria were not susceptible to plant extracts when compared to Gram-positive bacteria.

This is a sharp contrast to the obtained result for *Salmonella sp*. Although *Salmonella sp*. was Gram-negative bacteria, significant inhibition was observed when subjected to *Dioscorea alata* extract. These results were in contrast with those from previous screenings of medicinal plants for antimicrobial activity, where most of the active plants

showed activity against Gram-positive strains only (Rabe & Van Staden, 1997; Vlietinck et al., 1995).

The water extract from *in vivo* and *in vitro* samples of *Dioscorea alata*, effectively reduced the growth of all tested bacteria (Table 4.31). The water extract of *in vivo* (tubers) exhibited antimicrobial activity against gram-positive (*S. aureus*, *S. epidermis*) and gram-negative (*E. coli* and *Salmonella sp.*) bacteria showing zone of inhibition of 18.33 ± 0.88 mm, 23.00 ± 1.73 mm, 12.00 ± 0.58 mm and 18.00 ± 1.00 mm, respectively. Water extract of *in vitro* (callus) exhibited activity against *S. aureus*, *S. epidermis*, *E. coli* and *Salmonella sp.* with inhibition zone of 9.33 ± 0.67 mm, 10.67 ± 1.14 mm, 7.00 ± 0.58 mm and 10.00 ± 1.00 mm, respectively. Although *S .aureas* and *S. epidermis* belong to the same genus but their sensitivity to *Dioscorea alata* ethanolic extract was different. The difference between the inhibition zone of *Dioscorea alata* extracts against *S. aureas* and *S.e pidermis* showed that it has no selective effect on the genus *Staphylococci*.

The ethanolic and water extracts of *Dioscorea alata* showed different antibacterial activity. These results showed that the antibacterial activity and susceptibility test obtained in this study varied according to the extraction solvent used. Higher antibacterial activity was detected from ethanolic extracts against all bacteria tested, except *E .coli* for *in vivo* sample. For *in vitro* sample, ethanolic extract showed higher antibacterial activity against *E. coli* and *S .aureus*, and lower activity against *S. epidermis*, and *Salmonella sp.* compared to water extract. This observation was in agreement with the finding of Prakash & Hosetti (2010). Among all the tested extracts, ethanol proved to be the most potent bactericidal agent against all the strains as compared to other extracts. Similar result was also observed by Mello et al. (2010) whereby ethanol was more effective in extracting phenolic compounds than water and ethanol extract exhibited a higher antioxidant activity compared to the water extracts.

Variation in antibacterial activity of ethanolic and water extracts may probably be due to the type of bioactive compounds present in the different extraction solvents as suggested by Abiodun et al. (2007). According to Gberikon et al. (2015) the phytochemical analyses, saponins, tannins, steroids, phlobatannins, alkaloids, anthraquinones, and flavonoids were present in highest concentration in ethanol extract than water extract. These groups of compounds form the active principles that confer antibacterial activity on the plant. The water extract may either contain more non phenolic compounds or possess phenolic compounds that contain a smaller number of active groups than ethanol.

The antifungal activity was assayed against four filamentous fungi, which were *Penicillium sp.*, *A. niger*, *Fusarium sp.* and *Mucor sp*. The activity was evaluated using the agar disk diffusion method by measuring the diameter of the growth inhibition zone. In recent years, pressure to reduce the use of synthetic antifungal or fungicides in agriculture has increased. Concern have been raised about both the environmental impact and the potential health risk related to the used of these compounds. Some fungi can produce mycotoxins that have dangerous effects on human, animal and plant that can result in illness, death and economic loses. *A. niger* produce potent mycotoxins on foodstuffs and is most prevalent fungus effecting corn (Marassas, 1991). Some members of *Aspergillus* and *Fusarium* genera are well known to produce aflatoxins. These secondary metabolites are potent carcinogens, hepatotoxinx, teratogens and inmunosuppresive compounds (Ciegler, 1975). These fungi represent threats not only to the health of crops, but also to animals and humans ingesting contaminated feeds and foods (Quiroga et al., 2001). According to Aderiya (1996) the yam compounds were toxic to both vegetative and reproductive fungal structures in liquid media and on thin-layer chromatograms.

The ethanolic extract of *in vivo* (tubers) samples and *in vitro* samples (plantlets and callus) of *Dioscorea alata* subjected against *A. niger, Fusarium sp., Mucor sp.* and *Penilcilium sp*. were presented in Table 4.32. The analysis of crude extracts of *Dioscorea alata* showed inhibitory activity against fungi *Penicillium sp.* (Figure 4.35) and *Mucor sp*. (Figure 4.34). Ethanolic extract of tubers exhibited activity against *Penicillium sp.* and *Mucor sp* showing maximum zone of inhibition of 25.00 ± 1.15 mm and 10.33 ± 0.88 mm respectively. Ethanolic extract of plantlets exhibited activity against *Penicillium sp.* and *Mucor sp* showing zone of inhibition of 15.67 ± 2.40 mm and 7.67 ± 0.67 mm respectively. Ethanolic extract of callus exhibited activity against *Penicillium sp.* and *Mucor sp* showing zone of inhibition of 8.00 ± 0.58 mm and 7.33 ± 0.33 mm, respectively.

Results from Table 4.32 also showed that, ethanol (control negative) has no antifungal activity against *Mucor sp.* Extract from *in vivo* sample (tubers) and *in vitro* samples (plantlets and callus) inhibited mycelial growth of *Mucor sp*. and *Penicillium sp.* Sensitivity of tested fungi to the callus extracts was not as high as tubers and plantlets ethanolic extract. It was clear from the data shown in Table 4.32 that tubers displayed the highest antifungal activity in *Dioscorea alata*. Other studies of *Dioscorea sp*. also showed that tubers exhibit high antifungal activity. Sonibare & Abegunde (2012) detected, extract from tubers of *Dioscorea hirtiflora* have antifungal effect against *Penicillium sp.*

Ethanolic extract of *Dioscorea alata* (tubers, plantlets and callus) possess no activity against *A. niger* (Figure 4.32) and *Fusarium sp*. (Figure 4.33). The extracts did not inhibit the growth of *A. niger* and *Fusarium sp*., hence no visible zone of inhibition. In contrast, other reports showed that extract of *Dioscorea sp*. have significant activity against *A.niger*. Study by Sonibare & Abegunde (2012) showed *Dioscorea hirtiflora* and *Dioscorea dumetorum* have antifungal against *A. niger* but at high concentration of extraction, 50 mg/ml tubers extract of *Dioscorea deltoidea* have antifungal activity

against *A. flavus* and *A. parasiticus*, whilst 10 mg/ml have no effect (Chandra et al., 2013). Insufficient extract concentration from tubers, plantlets could callus may be the reason why no antifungal activity and no inhibition zone was observed in this study.

The water extract of *in vivo* sample (tubers) and *in vitro* sample (callus) of *Dioscorea alata* subjected against *A.niger, Fusarium sp., Mucor sp.* and *Penilcilium sp*. were presented in Table 4.33. Tubers and callus water extracts did not show any significant antifungal activity against all the tested fungi. The water extract of *Dioscorea alata* produced no inhibitory effect on the tested microorganisms, hence no visible zone of inhibition. Similar result have been reported where water extract had low or no antimicrobial activity (De Las Llagas, 2014). On the contrary, other studies revealed that high concentration of plant water extraction showed antifungal activity against *A. flavus* and *A. parasiticus* (Chandra et al., 2013). Inadequate of extract concentration to inhibit the fungi growth, may be the reason there were no inhibition zone observed in the present study of water extraction.

It was clear from the data shown that (Table 4.32), *Dioscorea alata* possess antifungal activity as shown in the current study. The ethanol extract showed the highest inhibitory effect on the test organisms while water extract did not inhibit any of the tested organisms. Differences in the extraction methods produced different type of chemical compounds. This fact enlightened the reason why in *in vivo* and *in vitro* water extracts there were no antifungal activity against *Penicillium sp.* and *Mucor sp* though ethanolic extract have. This is because ethanol extracted more phytochemicals that will inhibit growth of fungal as opposed to water solution. According to Dere et al. (1998), the differences in extraction rate and determination of the best solvent extraction might be influenced by different species or samples used. Despite these results, plant extracts obtained using organic solvents have limitations. The use of water as the extracting solvent is more favourable

than the use of organic solvents due to its environmental friendly and non-toxic characteristics.

In the present study, most of the results showed higher antimicrobial (antibacterial and antifungal) activity in *in vivo* samples of *Dioscorea alata* compared to *in vitro* samples. Antimicrobial activity differed in the *in vivo* and *in vitro* samples extracts probably due to inherent characteristic of the fully grown plants and the maturity of its chemically active constituents. Although the production of antimicrobial activity was lower in the *in vitro* samples, yet significant effect was observed and one of the *in vitro* ethanolic extract showed higher antibacterial effect against *E.coli* compared to *in vivo* extract. Grzegorczk et al. (2007) stated that, the compounds in *in vitro* plants had higher bioactivity than in *in vivo* plants.

The results also showed that antimicrobial activity in *in vivo* and *in vitro* extracts was lower compare to positive control and higher compare to negative control. Among all the tested extracts, ethanolic tubers extract proved to be the most potent bactericidal agent against all the strains as compared to water extracts, but it is not up to the standard drug chloramphenicol and carbendazim. According to Rauha et al. (2000), plants extracts generally contained flavonoids in glycosidic form. This maybe the reason why the plant extracts did not produce as marked inhibition as many of the positive control.

Antimicrobial growth inhibition detected in this study were due to the release of diffusible inhibitory compounds from the sample extracts. *Dioscorea alata* possess several medicinally important metabolites like allantoin (Wang et al., 2013), dioscorin (Hsu et al., 2002; Huang et al., 2007), anthocyanin, alatanin C (Yoshida et al., 1991), steroids (Araghinikam et al., 1996), alkaloids and beta-sitosterol. Allantoin has multitudinous bio-functions such as enhancing the growth of skin cell, anti-ulcer

medicine, anti-inflammatory medicine and antioxidant. Dioscorin was shown to inhibit angiotensin converting enzyme (ACE) those might be a potential for hypertension control. There are reports showing that alkaloids and flavonoids are responsible for the antifungal activities in higher plants (Cordell et al., 2001). Beta-sitosterol is antifungal component obtained from peels of *Dioscorea alata* tubers. Several species of *Dioscorea* are amongst the principle sources of diosgenin, which can be converted to medicinally important steroids (Van Staden & Fowlds, 1992). Recently, isoquinoline and isoquinuclidine alkaloids with antimicrobial effect were isolated from *Dioscorea dregeana* (Mulholland et al., 2002). These compounds may also cause the inhibition of bacterial growth in the present study in *Dioscorea alata.* Therefore, the presence of these phytochemicals, could justify the observed antifungal activities in the current study.

Interestingly, in the present study, antimicrobial agents present in *in vivo* sample (tubers) also detected in *in vitro* samples. This result confirms that, secondary metabolite produced in intact plants of *Dioscorea alata* also produced under *in vitro* conditions. According to Ramachandra & Ravishankar (2002), plant cells are biosynthetically totipotent, which means that each cell in culture retains complete genetic information, hence, is able to produce the range of chemicals found in the parent plants. Furthermore, manipulation of the culture environment such as culture media, nutrients levels, plant growth regulators, stress factors and light were able to stimulate formation, enhancement and accumulation of secondary metabolites *in vitro*. Fujita (1990) suggested that *in vitro* culture can be alternative methods for synthesis of secondary metabolites which are commercially essential for the production of drugs, flavours, perfumes and pigments that are often difficult to synthesize chemically.

CHAPTER 6: CONCLUSION

Dioscorea alata L. is capable of producing bioactive compounds, which can play an important role in maintaining human health. However, seasonal and environmental variations, soil types and plant age associated with difficulties in the reproduction and propagation of this species which limits the availability of many bioactive compounds. Tissue culture offers an opportunity to exploit the cells, tissues, organs or entire organisms by growing them under *in vitro* conditions to mass propagate this plant and produce the chemical compounds desired. This is because plant cells are biosynthetically totipotent, which means that each cell in culture retains complete genetic information of the mother plant, hence the explant is able to regenerate into complete plantlets rely on the fact that many plant cells have the ability to regenerate into a whole plant. Besides, explants also able to produce the range of chemical compounds found in the mother plant as observed by many researchers, for example Ramachandra & Ravishankar (2002). Little studies concerning tissue culture has been done on *Dioscorea alata*, therefore, the present work reported in greater details on *in vitro* studies of *Dioscorea alata* together with other biotechnological studies, synthetic seeds, biological activities (antimicrobial) and secondary metabolite production *in vitro*.

An efficient and reliable plant regeneration protocol was established for *Dioscorea alata* in the current work. The method developed using leaf, node and stem as the explants is simpler and more reliable since the vines are easily propagated *in vitro* all year round and hence there is no limitation of planting materials or explants. Node was the best explant for direct shoots and roots formation. MS medium supplemented with 1.0 mg/l BAP and 0.5 mg/l NAA was the optimum medium for shoots formation from node explants. Through tissue culture, *Dioscorea alata* can be mass propagated throughout the year, not depending on tubers only as planting materials.

Formation of microtubers from regenerated plantlets is an advantage. Microtubers contained same metabolite compounds as the mother plant and can serve as the planting materials. In this study it was demonstrated that the micropropagation of *Dioscorea alata* using microtubers is possible with no dormancy period was observed. Micrortubers germinated after 2 weeks in culture produced shoots and roots. Microtubers offer several advantages over *in vitro* regenerated plantlets, since they can be stored for a long period and transplanted directly into the field without acclimatization proses. The micropropagation technique described here may be highly useful for raising disease free quality planting materials of *Dioscorea alata* for commercial and off season cultivation which will help in reducing the quantity of ware tubers reserved for use as seed tuber.

The present study also focused on callus production in *Dioscore alata*. All the explants used, leaf, node and stem were successfully induced to produce purplish coloured callus after being cultured onto callus induction media. Effects of plant growth regulators BAP and NAA, liquid or solid media, additional activated charcoal, additional myo-inositol and photoperiod were investigated on callus formation and proliferation. It was found that liquid media was the best for coloured callus production with better aeration. Activated charcoal is commonly used in tissue culture media. It is an essential component of many plant tissue culture media which prevents browning of cultured tissues and media by adsorption of toxic compounds like polyphenols released by cultured tissues. However, result from this current experiment showed that, activated charcoal did not only absorb phenolic compounds released from wounded explants but also absorb nutrients needed for callus formation in *Dioscorea alata*. Thus, applying activated charcoal in callus inducing media of *Dioscorea alata* are not so suitable although there is no harmful effect. In the current study, results indicated that there was no significant differences for all the treatments in term of percentage of explants produced callus. However, maximum callus growth was obtained on culture medium supplemented with additional myo-inositol.

Thus, MS supplemented with 2.0 mg/l 2,4-D and 0.5 mg/l myo-inositol cultured in liquid medium was the optimum medium for coloured callus induction. The current experiments demonstrated that light regime did not significantly influenced callus growth. However, different colours of callus were observed in different photoperiod treatment.

A method for indirect plant regeneration through somatic embryos was also developed from node explants of *Dioscorea alata*. Recent study shows *Dioscorea alata* can be regenerated via somatic embrygenesis. Embryogenic callus formation was induced and differentiated from non embrogenic callus using Double staining technique. After that, embryogenic callus were subculture on somatic embryos induction media. The highest average number of somatic embryos (23.27 ± 0.14) was detected in MS medium supplemented with 2.0 mg/l 2-4,D and 1.0 mg/l BAP. The embryos at globular stages underwent sequential development stages then germinated one month after being transferred in germination media. The first sign of somatic embryo maturation was the formation of cotyledonary collar. Hook type of germination was observed from the cotyledonary collar. Tiny leaves like hook started to emerge from the opening of the collar. The highest percentage, (60.00±0.09) of embryos germination was observed on MS medium supplemented with 1.0 mg/l BAP.

There is currently much interest in creating synthetic seeds possessing outer protective coatings, thereby bestowing the vegetative tissue with the storage and handling qualities of natural seeds. Formation of synthetic seed in *Dioscorea alata* was achieved by complexation of a 3.0 % solution of sodium alginate with 100 mM calcium chloride for 30 min thereby forming an insoluble gel matrix of calcium alginate. The current study confirms that this synthetic seed can be regenerated into complete plantlets. However, germination of synthetic seeds was influenced by several factors such as the type of propagules encapsulated, encapsulation matrix and germination media. Node segments

encapsulated with 3.0 % sodium alginate hardened in 75 and 100 mM Calcium chloride dehydrate fortified with 1.0 mg/l BAP singly and 1.0 mg/l BAP combination with 2.0 mg/l 2,4-D is the optimum for synthetic seed production in *Dioscorea alata* with 100 ± 0.00 % germination rate in liquid medium.

Micropropagation of *Dioscorea alata* was considered successful only when the plantlets attained from tissue culture (*in vitro* environment) was transferred and survived in the natural or *ex vitro* environment. Sowing media for acclimatization were analysed for their texture and fertility content. Black soil and Red soil was selected as the growth media because of their texture and content are suitable for *Dioscorea alata* growth. Acclimatization process was then carried out step by step. Plantlets of *Dioscorea alata* were positively adjusted with the new environment which is very different from the *in vitro* environment. A significant percentage of survival, (93.33 ± 0.05 %) was obtained in mixture of Black soil and Red soil after one month of acclimatization. After 2 months of acclimatization, the survival rate was declined to 90.00 ± 0.06 %. The micropropagated plantlets grew normally without any macromorphological variation between *in vivo*, *in vitro* and acclimatized plantlets.

Besides *in vitro* regeneration, the present study demonstrated the possibility of production of secondary metabolites in *in vitro* plantlets and callus. In *in vivo* and *in vitro* samples of *Dioscorea alata* contained both phenolic compounds and flavonoids. The total phenolic content of *in vivo* sample was 73 mg 100 g^{-1} and *in vitro* sample was 52 mg 100 g^{-1} whilst total flavonoids of *in vivo* sample was 48 mg 100 g^{-1} and *in vitro* sample was 21mg 100 g^{-1}. Tubers of *Dioscorea alata* have vivid purple colour which are verified as anthocyanin pigment. In addition to their ability to provide vibrant purple colour, they are widely acknowledged as having significant health-giving properties as an antioxidant, anti-inflammatory, anti-ulcer and wound healing properties. Comparative study of

anthocyanin content in *in vivo* and *in vitro* samples of *Dioscorea alata* was carried out in the present study. *In vivo* and *in vitro* samples of *Dioscorea alata* both contained anthocyanin properties with higher anthocyanin content observed from *in vivo* samples. Callus culture has been successfully applied for the production of plant secondary metabolites. However, media composition and culture conditions have to be optimized for an intensive biomass increase and the accumulation of desired metabolites. Therefore, in the present study light condition (photoperiod) was manipulated to increase anthocyanin content in callus cultures of *Dioscorea alata*. The results showed that callus exposed to different photoperiods produce different amount of anthocyanin content. The highest anthocyanin content was 295.21 ± 0.20 mg cya-3-glu / 100 g FW detected in coloured callus maintained at 16L/8D photoperiod.

All plants produced their own phytochemical substances especially to protect against some particular microorganisms. Therefore, *in vivo* and *in vitro* samples of *Dioscorea alata* were tested for their antibacterial properties against *S. aureus*, *S. epidermis*, *E. coli* and *Salmonella sp*. and antifungal activities against *Penicillium sp*., *A. niger*, *Fusarium sp*. and *Mucor sp*. The activity was evaluated using the agar disk diffusion method by measuring the diameter of the growth inhibition zone. Extracts of both *in vivo* and *in vitro* samples exhibited antibacterial activity against all bacteria tested. For antifungal activity, inhibition zone was observed against *Penicillium sp*. and *Mucor sp*. Even at low concentrations, (20 µl) *Dioscorea alata* contained potent antimicrobial and antifungal activity nearly equal to that of the commercial antibiotic and fungicide. Ethanolic extract of the test plant, *Dioscorea alata* possess more antimicrobial activity as opposed to water extract. Ethanolic extract was found to be more potent than the water extract against *E. coli, S. aureus*, *S. epidermis*, and *Salmonella sp.*, *Penicillium sp.* and *Mucor sp*.

The findings of the current study would be useful for farmers and plant breeders in producing better planting materials for *Dioscorea alata* cultivation not solely depends on tubers as planting materials. The findings also suggested that by consuming *Dioscorea alata* can replace the dependency on synthetically-produced supplementary health products for whom looking for healthy food crops with high medicinal value and anthocyanin content. Due to the high ability in regeneration and multiplication, the plant regeneration system of *Dioscorea alata* established in the present study will be efficiently used for rapid clonal propagation. This study of tissue culture of *Dioscorea alata* L. was considered a success and the findings of these studies hopefully can be shared with others in similar field. For future work, cellular behavior studies comparing cells *in vivo and in vitro* can aid in understanding regenerative potential of this species and same aspect of molecular work should be undertaken to obtained better hybrid of this species.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

PUBLICATIONS

1. Mahmad, N., Taha, R.M., Othman, R., **Abdullah, S**., Anuar, N., Elias, H. & Rawi, N. (2018). Anthocyanin as potential source for antimicrobial activity in *Clitoria ternatea* L. and *Dioscorea alata* L. *Pigment and Resin Technology*, *47*(6), 490-495.

2. Elias, H., Taha, R.M., Hasbullah, N.A., Othman, R., Mahmad, N., Azani, S. & **Abdullah, S**. (2018). Detention and quantification of natural pigments extracted from callus of *Echinocereus cinerascens*. *Pigment and Resin Technology*, *47*(6), 464-469.

3. Anuar, N., Mahmad, N., Wafa, S.N., **Abdullah, S**. & Elias, H. (2017). Effects of zeatin and gibberellic acid on regeneration and *in vitro* flowering of *Phlox paniculata* L. *Maejo International Journal of Science and Technology*, *11*(2), 115-125.

PAPERS PRESENTED

1. **Abdullah, S**., & Taha, R.M. (2017). Effect of light on anthocyanin accumulation in callus cultures of *Dioscorea alata* L. Paper presented at the 6th International Conference on Functional Materials & Devices (ICFMD 2017), Melaka, Malaysia. 15-18th August 2017.

2. **Abdullah, S**., & Taha, R.M. (2016). Callus induction and *in vitro* plant regeneration of *Dioscorea alata* L. Paper presented at the ISER 21th International Conference, Taipei, Taiwan. 16th Feb 2016.

3. **Abdullah, S**., & Taha, R.M. (2015). Comparative study of antioxidant activity in *in vivo* and *in vitro* sample of purple greater yam (*Dioscorea alata* L.). Paper presented at the 17th International Conference on (2015), Madrid, Spain, 12-13 November 2015.

Anthocyanin as potential source for antimicrobial activity in *Clitoria ternatea* L. and *Dioscorea alata* L.

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Abstract

Purpose – The purpose of this paper is to validate the antimicrobial activity (both antibacterial and antifungal) of *in vivo* and *in vitro* ethanolic anthocyanin extracts of *Clitoria ternatea* L. (vivid blue flower butterfly-pea) and *Dioscorea alata* L. (purple yam) against selected bacteria (*Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*) and fungi (*Fusarium sp*., *Aspergillus niger* and *Trichoderma sp*.).

Design/methodology/approach – The freeze-dried samples (0.2 g) from *in vivo* vivid blue flowers of *C. ternatea* L. were extracted using 10 mL ethanol (produced ethanolic red extraction) and 10 mL distilled water (produced aqueous blue extraction) separately. Two-month-old *in vitro* callus samples (0.2 g) were only extracted using 10 mL ethanol. The anthocyanin extractions were separated with the addition (several times) of ethyl acetate and distilled water (1:2:3) to remove stilbenoids, chlorophyll, less polar flavonoids and other non-polar compounds. Furthermore, the antimicrobial properties were determined using agar diffusion technique. Three bacteria (*B. subtilis*, *S. aureus* and *E. coli*) and fungi (*F. sp*., *A. niger* and *T. sp*.) were streaked on bacteria agar and dextrose agar, respectively, using "hockey stick". Then, the sterile paper discs (6 mm diameter) were pipetted with 20 μL of 1,010 CFU/mL chloramphenicol (as control for antibacterial) and carbendazim (as control for antifungal) *in vivo* and *in vitro* extracts. The plates were incubated at room temperature for 48 h, and the inhibition zones were measured.

Findings – Based on the results, both *in vivo* and *in vitro* ethanolic extracts from vivid blue flowers of *C. ternatea* L. showed the best antibacterial activity against the same bacteria (*B*. subtilis), 11 and 10 mm inhibition zones, respectively. However, different antifungal activity was detected in *in vitro* ethanolic callus extract (12 mm), which was against *T. sp*., contrary to *in vivo* ethanolic extract (10 mm), which was against *F. sp*.; antibacterial activity of *D. alata* L. was seen against the same bacteria (*E. coli*) with the highest inhibition zone for *in vivo* extract (8.8 mm), followed by *in vitro* extract (7.8 mm).

Research limitations/implications – Anthocyanins are responsible for the water soluble and vacuolar, pink, red, purple and blue pigments present in coloured plant pigments. These pigments (pink, red, purple and blue) are of important agronomic value in many crops and ornamental plants. However, anthocyanins are not stable and are easy to degrade and fade whenever exposed to light.

Social implications – Plant extracts containing bioactive agents with antimicrobial properties have been found to be useful in treating bacterial and fungal infections, as well as showed multiple antibiotic resistance.

Originality/value – Both *in vivo* and *in vitro* extracts from vivid blue flower petals (*C. ternatea* L.) and purple yam (*D. alata* L.) have important applications as natural antimicrobial (antibacterial and antifungal) agents in the coating industry, instead of natural pharmaceutical products.

Keywords Antibacterial, *Clitoria ternatea* L., *Dioscorea alata* L., Antifungal, Ethanolic extract, Inhibition zone

Paper type Research paper

Introduction

C. ternatea L. (vivid blue flower butterfly-pea), or locally known as "Pokok Bunga Talang" in Malaysia, is used as a natural blue colourant in "Nasi kerabu" preparation. Meanwhile, this species is known worldwide as a medicinal plant that possesses pharmacological activities including antimicrobial, anti-oxidant, anti-inflammatory, antipyretic, anti-helminthic and analgesic activities (Mukherjee *et al.*, 2008; Gupta *et al.*, 2010). The vivid blue flower of *C. ternatea* L. contains dietary anthocyanins (Terahara *et al.*, 1996), as well as *Dioscorea alata* L. (purple yam), which is widely cultivated in Asia, Pacific Islands and Africa

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(Mignouna *et al.*, 2003) including Malaysia, which is called "Ubi Badak", with big purple tubers that grow underground. Anthocyanins are responsible for the water soluble and vacuolar pink, red, purple and blue pigments present in coloured plant pigments. These pigments (pink, red, purple and blue) are of important agronomic value in many crops and ornamental plants. However, anthocyanins are not stable and are easy to degrade and fade whenever exposed to light.

Naturally, most microorganisms are found in soil, which are very important in providing plants with gas and minerals (decomposers). Pathogenic bacteria (*Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli)*, being the highest number of microorganisms on top of the soil (up to 15 cm depth), play an important role in gas cycles (such as nitrogen fixation), while fungi (*Fusarium sp.*, *Candida albicans* and *Trichoderma sp.*) decay organic substances that add cellulose and inorganic substances into the soil. Pietikäinen *et al.* (2000) reported that the optimum growth for bacteria and fungi occurs at 25-30°C and is responsible for causing infections to plants (losses in agricultural industry), animals and humans (can cause diarrhoea and skin infection).

Recently, tissue culture studies have focussed on comparing antimicrobial activities of *in vivo* (such as leaf, stem, root and flower) and *in vitro* (callus and regenerated plant) sources to be introduced as multipurpose plant applications, which could be beneficial to mankind, instead of mass propagation. Under sterile condition, *in vitro* callus or regenerants may contribute natural sources for pharmaceuticals, food supplements, cosmetics and safety colourant production. In plant tissue culture, different concentrations and combinations of auxins and cytokinins have different effects on the growth of *in vitro* explants. A balance between auxin and cytokinin growth regulators is most often required for the formation of adventitious shoot and root meristems. Therefore, plant extracts containing bioactive agents with antimicrobial properties have been found to be useful in treating bacterial and fungal infections (Adeniyi *et al.*, 2010), as well as showed multiple antibiotic resistance (Kuete *et al.*, 2012).

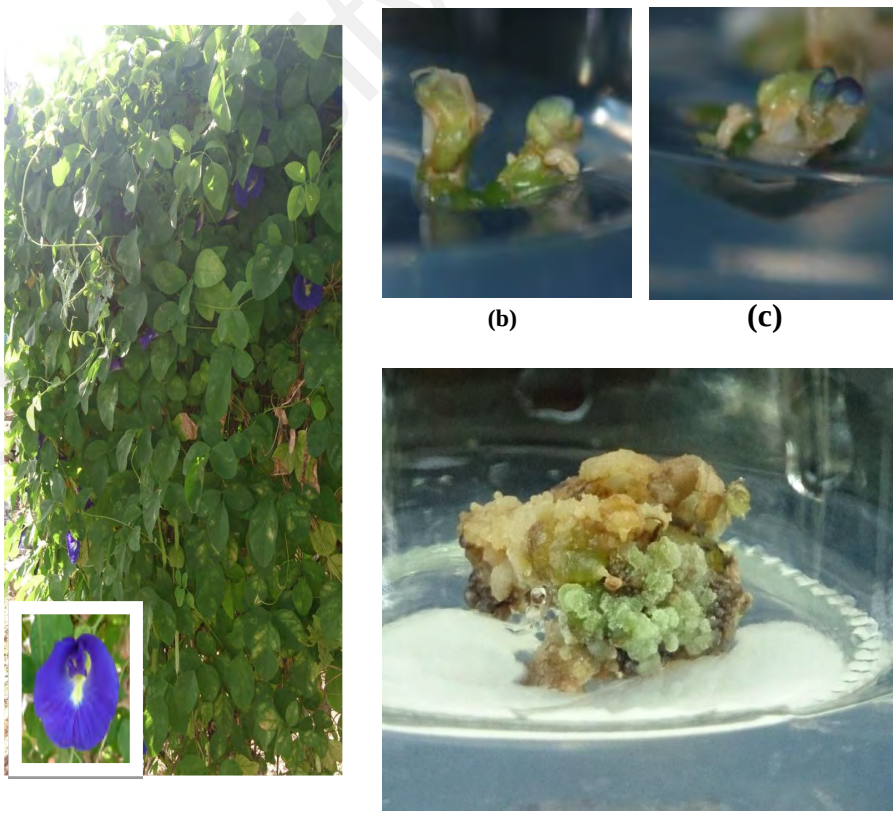
The main objective of this research was to investigate the antibacterial and antifungal activities of *in vivo* and *in vitro* ethanolic anthocyanin extracts of *C. ternatea* L. and *D. alata* L. against selected bacteria (*B. subtilis*, *S. aureus* and *E. coli*) and fungi (*F. sp.*, *Aspergillus niger* and *T. sp.*), which can be potentially introduced as natural antimicrobials for coating production.

Materials and methods

In vivo plant samples

Petals of the vivid blue flower (*C. ternatea* L.) and purple yam (*D. alata* L.) were obtained from the garden of Institute of Biological Sciences, University of Malaya, Kuala Lumpur,

Figure 1 *In vitro* callus formation of *C. ternatea* L. from its explant cultured on Murashige and Skoog media supplemented with 40 mg/L $ADSO_4$ + 2 mg/L NAA + 2 mg/L BAP (a) *in vivo* plant of *C. ternatea* L.; (b) two-week-old *in vitro* flower explant; (c) flower explant with vivid blue petals; and (d) two-month-old green callus



Malaysia. The samples were sealed in an aluminium foil and kept at −80 ± 1°C and later freeze-dried for 72 h. Then, the dried samples were crushed into a powdered form and kept at −20 ± 1°C until needed.

In vitro plant samples (callus)

Originally, *in vivo* plant samples were sterilised using 90 per cent (v/v) chlorox and 70 per cent (v/v) ethanol, followed by rinsing three times with distilled water. Under aseptic condition in a laminar flow, these samples were cut (3-5 mm^2) and cultured on Murashige and Skoog (MS) media (1962) supplemented with plant hormones, including adenine hemisulfate ($ADSO_4$), naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP), for callus induction. After eight weeks, the callus was collected and freeze-dried for 72 h.

Sample extraction

The method for sample extraction was carried out following the description given by Rahiman *et al.* (2013) with minor modification. The freeze-dried samples (0.2 g) were soaked in 10 mL ethanol for 24 h at room temperature under dark conditions to avoid degradation of anthocyanin compound. The mixtures were then centrifuged at 5,000 rpm for 10 min. Subsequently, rotary evaporator was used to remove alcohol in the solution by evaporation under reduced pressure at a relative low temperature (<30°C). Finally, anthocyanin extraction was separated with the addition (several times) of ethyl acetate and distilled water (1:2:3) to remove stilbenoids, chlorophyll, less polar flavonoids and other non-polar compounds.

Antimicrobial test (paper disc diffusion method)

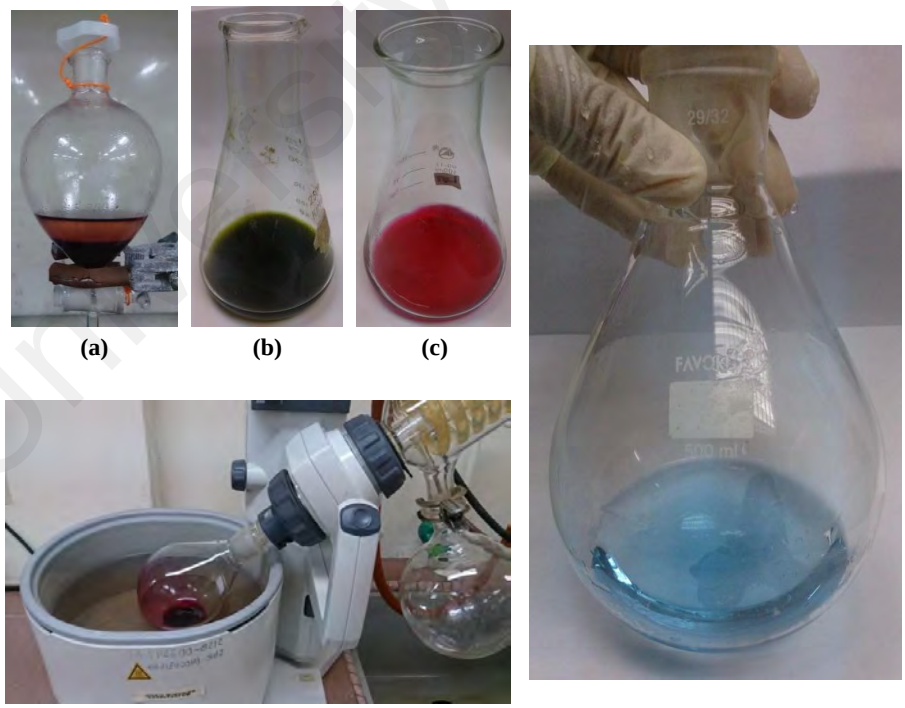
Antimicrobial properties were determined using the agar diffusion technique (Bauer *et al.*, 1966). Three bacteria (*B. subtilis*, *S. aureus* and *E. coli*) and fungi (*F. sp.*, *A. niger* and *T. sp.*) were streaked on bacteria agar and dextrose agar, respectively, by using a "hockey stick". Then, the sterile paper discs (6 mm diameter) were pipetted with 20 μL of chloramphenicol (as control for antibacterial), carbendazim (as control for antifungal) *in vivo* extracts and *in vitro* extracts. The controls were prepared in 0.1 mg/L and 10^{10} CFU/mL. The plates were incubated at room temperature for 48 h, and the inhibition zones were measured. Antimicrobial agents were recognised by the formation of inhibition zones, which kill or inhibit the growth of microorganisms (Jagessar *et al.*, 2008). Three replicates were used for each microorganism tested.

Results and discussion

Antibacterial and antifungal activities of anthocyanin extraction from vivid blue flower of C. ternatea L.

Generally, the anthocyanins from petals of *C. ternatea* L. (Figures 1 and 2) exhibited a potent inhibitory activity against all the tested bacteria (*B. subtilis, S. aureus* and *E. coli*) and fungi

Figure 2 Extracts of *C. ternatea* L.



Notes: (a) Separation of anthocyanin extract; (b) green ethanolic extract from *in vitro* callus; (c) red ethanolic extract from *in vivo* flowers; (d) rotary evaporation to remove alcohol from extractant; (e) blue aqueous extract from flowers

(*F. sp.* and *T. sp.*), but *A. niger* was the only one that responded to *in vitro* callus extract. Moreover, *in vitro* callus extract was found to give better inhibition zone (8-11 mm) as compared to *in vivo* ethanolic flower extract, which produced 7-10 mm inhibition zone. These anthocyanin compounds are shown in Figure 3. According to Grzegorczyk *et al.* (2007), the compounds in *in vitro* extracts had higher bioactivity than those in *in vivo* extracts. However, no inhibition zone was observed for all tested bacteria and fungi for *in vivo* aqueous flower extract. Thus, the natural blue colourant that was extracted with water did not exhibit antibacterial and antifungal properties.

For antibacterial activity (Table I and Figure 4), both *in vivo* and *in vitro* ethanolic extracts from petals of *C. ternatea* L. showed the best antibacterial activity against the same bacteria (*B. subtilis*), 11 and 10 mm inhibition zones, respectively. Chloramphenicol (as control) reacted against *S. aureus* with an inhibition zone of 28 mm.

However, for antifungal activity (Table II and Figure 5), different antifungal activities were detected for *in vitro* ethanolic callus extract (12 mm) against *T. sp.*, contrary to *in vivo* ethanolic extract (10 mm) against *F. sp.* Carbendazim (as control) inhibited the growth of *T. sp.* (28 mm); the same was shown by *in vitro* ethanolic callus extract. Naturally, plants can produce antifungal compounds to protect themselves from biotic attack that could be essential for fungi infection resistance.

Generally, antimicrobial activity differs in *in vivo* and *in vitro*, probably because of the inherent characteristics of the fully grown plants and maturity of its chemically active constituents. In addition, antimicrobial activity of the callus extract can vary between differentiated and undifferentiated plants cells, depending on the biocompound production.

C. ternatea L. is known as a medicinal plant and is widely used for treating eyes, throat and skin infections and ulcers. In addition, it can be used as a phytochemical substance. The phytochemical investigations revealed the presence of saponins, carbohydrates, alkaloids, proteins, anthroquinones and phytosterols. It is used as a diuretic, antihelmintic, antidiabetic, antipyretic and brain tonic. All parts of *C. ternatea* L. contain peptides called cliotides that have potent antimicrobial properties against *E. coli*. Therefore, because of the increasing demand for medicinal and nutritive forage purposes of *C. ternatea* L., the US Development Agency (USDA, 2014) intends to conserve *C. ternatea* L., along with 16 other leguminous species, with potentially useful phytochemicals (Morris, 1999).

Figure 3 Anthocyanin extraction from *C. ternatea* L.

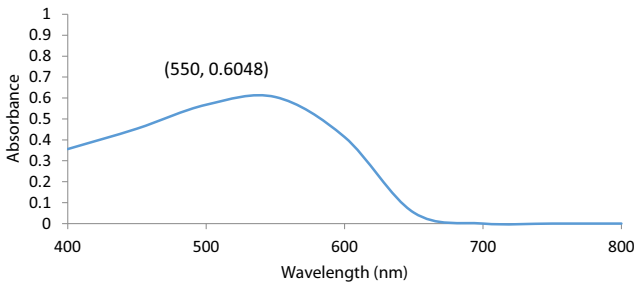


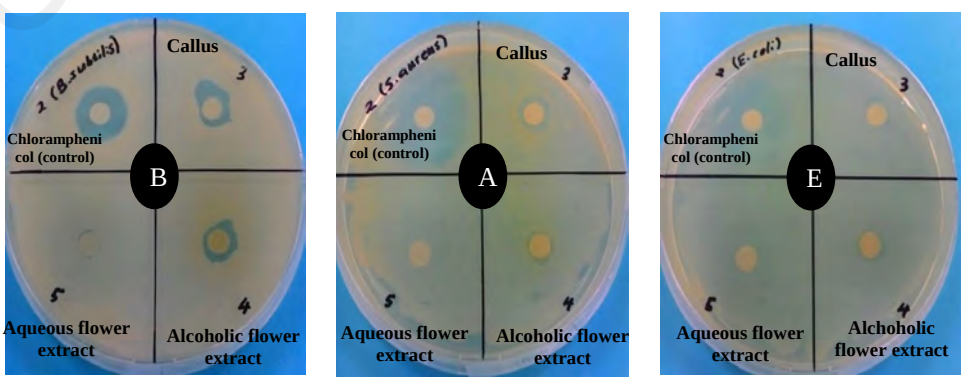
Table I Antibacterial activities of *C. ternatea* L. against *B. subtilis*, *S. aureus* and *E. coli*

	Mean inhibition zone diameter, mm			
Bacterial (10^{10} CFU/mL)	Chloramphenicol (0.1 g/100 mL)	*In vitro* alcoholic callus extract	*In vivo* alcoholic flower extract	*In vivo* aqueous flower extract
B. subtilis	17.0 ± 0.3	11.0 ± 0.3	10.0 ± 0.3	0
S. aureus	28.0 ± 0.6	10.0 ± 0.0	7.0 ± 0.7	0
E. coli	20.0 ± 0.3	8.0 ± 0.6	8.0 ± 0.5	0

Antibacterial activity from anthocyanin extracts of *D. alata* L. (purple yam)

Generally, antibacterial activity (Table III and Figure 6) of *D. alata* L. (Figure 7) was seen against the same bacteria (*E. coli*) with the highest inhibition zone from *in vivo* extract (8.8 mm), followed by *in vitro* extract (7.8 mm). Chloramphenicol (as control) showed inhibition response against *E. coli* (6.8 mm). According to Moriya *et al.* (2015), new acylated anthocyanins were found in *D. alata* L.

Figure 4 Antibacterial activity from explant of *C. ternatea* L. against (a) *B. subtilis*, (b) *S. aureus*, and (c) *E. coli*

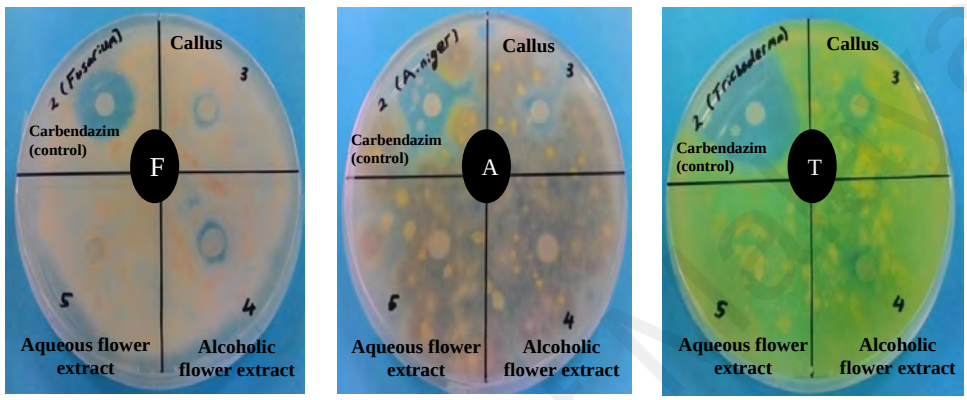


(a) (b) (c)

Table II Antifungal activities of *C. ternatea* L. against *F. sp*., *A. niger* and *T. sp*

Fungi (10^{10} CFU/mL)	Carbendazim (0.1 g/100 mL)	Mean inhibition zone diameter, mm		
		In vitro alcoholic callus extract	*In vivo* alcoholic flower extract	*In vivo* aqueous flower extract
F. sp.	20.0 ± 0.4	9.0 ± 0.7	10.0 ± 0.6	0
A. niger	17.0 ± 0.6	10.0 ± 0.2	0	0
T. sp.	28.0 ± 0.3	12.0 ± 0.3	8.0 ± 0.5	0

Figure 5 Antifungal activity from flower explant of *C. ternatea* L. against (a) *F. sp*.; (b) *A. niger*; and (c) *T. sp*.

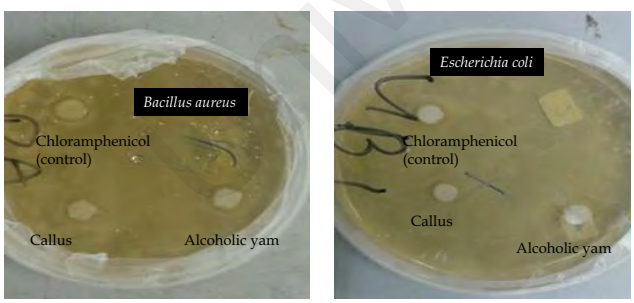


(a) **(b)** **(c)**

Table III Antibacterial activities of *D. alata* L. against *B. subtilis* and *E. coli*

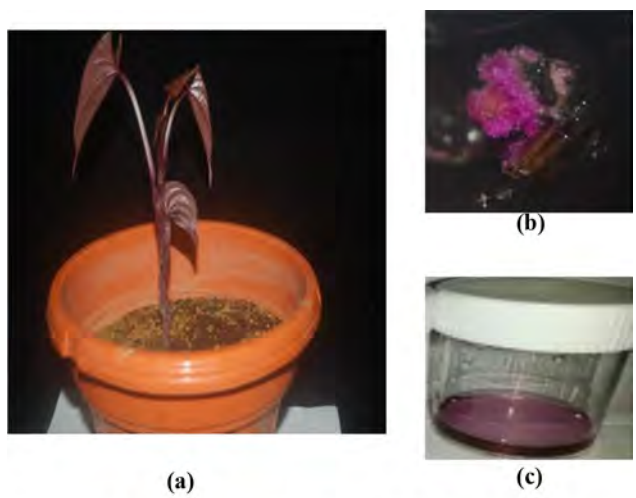
Bacterial (10^{10} CFU/mL)	Mean inhibition zone diameter, mm		
	Chloramphenicol (0.1 g/100 mL)	*In vitro* alcoholic callus extract	*In vivo* alcoholic yam extract
B. subtilis	6.5 ± 0.0	6.2 ± 0.1	6.6 ± 0.0
E. coli	6.8 ± 0.1	7.8 ± 0.1	8.8 ± 0.0

Figure 6 Antibacterial activity of *D. alata* L. against (a) *B. aureus* and (b) *E. coli*



(a) **(b)**

Figure 7 *D. alata* L.



(a) **(b)** **(c)**

Notes: (a) Dark red coloured of *in vivo* plant; (b) purple *in vitro* callus formed on Murashige and Skoog media supplemented with 2.0 mg/L NAA; (c) purple alcoholic extract of tuber

Conclusions

Based on the results, both *in vivo* and *in vitro* ethanolic extracts of vivid blue flowers of *C. ternatea* L. showed the best antibacterial activity against the same bacteria (*B. subtilis*), 11 and 10 mm inhibition zones, respectively. However, a different antifungal activity was detected in *in vitro* ethanolic callus

extract (12 mm), which was against *T. sp.*, contrary to *in vivo* ethanolic extract (10 mm), which was against *F. sp.*, and antibacterial activity of *D. alata* L. was seen against the same bacteria (*E. coli*) with the highest inhibition zone for *in vivo* extract (8.8 mm), followed by *in vitro* extract (7.8 mm). This study revealed that natural anthocyanin extracts from vivid blue flowers (*C. ternatea* L.) and purple yam (*D. alata* L.) can be potentially used in coating technology against bacteria and fungi as they exhibit antimicrobial properties.

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Detection and quantification of natural pigments extracted from callus of *Echinocereus cinerascens*

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Abstract

Purpose – This paper aims to study the effect of different organic solvents on the extraction of pigments present in callus cultures of *E. cinerascens*.

Design/methodology/approach – Attempts have been made to extract pigments from callus cultures through tissue culture system as an alternative replacement for conventional plant cultivation as tissue culture provides unlimited supplies of plant samples. Callus of *E. cinerascens* was induced from stem explant cultured in Murashige and Skoog medium supplemented with combination of 0.5 mg/L 6-benzylaminopurine and 0.5 mg/L α-naphthaleneacetic acid maintained under photoperiod of 16 h light and 8 h dark. Fresh samples of the callus were harvested and dissolved in various types and concentrations of solvents such as 100 per cent acetone, 80 per cent acetone, 95 per cent ethanol, 100 per cent methanol and 90 per cent methanol. Each of the mixtures was directly centrifuged to get clear supernatant containing pigments of interest. The pigments were detected and subsequently quantified via two simple techniques, ultraviolet-visible (UV-Vis) spectrophotometer and thin layer chromatography (TLC).

Findings – UV-Vis spectrophotometer detected two families of pigments present in the callus cultures, namely, carotenoids (carotene and xanthophyll) and tetrapyrroles (chlorophyll *a* and *b*). Pigment contents in various solvent extractions were estimated using spectroscopic quantification equations established. Through TLC, spots were seen on the plates, and Rf values of each spots were assessed to indicate the possible existence of carotenoids and tetrapyrroles.

Originality/value – This preliminary study offers significant finding for further advance research related on natural pigments extracted from *E. cinerascens* that would provide profits in the future applications, especially in food industry, medicine, agriculture, etc.

Keywords Pigments, Carotenoids, Chlorophylls, Thin layer chromatography (TLC), UV-Vis spectrophotometer

Paper type Research paper

Introduction

Pigments are molecules or chemical compounds that absorb specific wavelengths of light and reflect only certain wavelengths of visible light. In plants, pigments are essential to undergo photosynthesis, one of the earliest biological processes to evolve, which provided evidence for the significance of pigments in plant functions (Ustin *et al.*, 2009). Pigments can be grouped in a few different classes (Mortensen, 2006; Schoefs, 2004; Hasni *et al.*, 2011; Schoefs, 2002), namely, tetrapyrroles (e.g. chlorophylls), carotenoids (e.g. carotenes),

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flavonoids (e.g. anthocyanins) and alkaloids (e.g. betalains). Chlorophylls are the most abundant pigments in plants which promote green colour and are lipid soluble. Generally, there are two types of chlorophylls, i.e. chlorophyll a and chlorophyll b, which only differ in the replacement of the tetrapyrrole ring or the presence of –CH3 instead of –CHO. Besides, carotenoids are also lipidsoluble but represent yellow, orange and red colours. Typically, there are two carotenoids that are commonly identified, namely, beta carotene and xanthophylls. Meanwhile, other pigments such as anthocyanins and betalains are water soluble. Both are chemically related but anthocyanins show blue, purple, red and orange colours, while betalains exhibit yellow, red and purple colours.

Nowadays, research concerning plant pigments is highly in demand due to its usefulness and potential applications in food or textile industry (natural colourant), cosmetic or medicine (antioxidant and anticancer), agriculture (fertilizer and crop protection) and so on. Natural colourant is applied extensively in the food industry as one of a viable alternative to replace synthetic food colourant, which was reported unsafe. Plant pigments have been applied as natural food colourants (Mortensen, 2006) and are suggested to be beneficial to human health (Sun *et al.*, 2005). Additionally, it has been discovered that plant pigments play important roles in preventing human diseases (Glenn and Pablo, 1995; Lanfer-Marquez *et al.*, 2005; Rao and Rao, 2007; Sergio *et al.*, 1999). Production of plant pigments through conventional plant cultivation is limited due to low supply of raw materials (Schoefs, 2004). This problem leads to discovery of new procedures for production of plant pigments via tissue culture or other biotechnology methods. Tissue culture provides effective system in production of pigments as the plant samples can be obtained in industrial scale in just a short period. Through this technique, not only quantity but also quality of the samples could be enhanced by modifying the specific nutrients in the medium supplied. Consequently, this would influence the production of desirable chemical compounds in the samples. Indeed, the pigments could be obtained from callus samples without destroying the plants. Seemingly, tissue culture is widely used to isolate compounds or secondary metabolites for new drug development and medicine (Ahmed *et al.*, 2010).

Previous studies have indicated that various methods have been developed to detect pigments. Nevertheless, in the present study, the pigments extracted from callus cultures of *Echinocereus cinerascens* (hedgehog cactus) were detected through two simple methods, namely, ultraviolet-visible (UV-Vis) spectrophotometer (spectroscopic method) and thin layer chromatography (TLC; chromatographic method). Spectroscopy is a classical method that usually permits crude identification of the pigments present in an extract. In addition, major pigments can be depicted by the absorbance spectrum. Even though this is the simplest method used for pigment detection, it is less efficient, especially for the plant sample that contains more than three pigments (Schoefs, 2002) as there is a strong limitation due to overlapping of the absorbance readings. Hence, chromatographic method, i.e. TLC, is one of the accessible methods that facilitate separation of four pigments, which has been recognised as a fast, effective and relatively cheap method. TLC has been widely used in laboratories for chemical analysis of agricultural products and plants (Sherma, 2000). Furthermore, TLC and high-performance liquid chromatography (HPLC) are the recommended methods to detect and analyse the pigments as these compounds are non-volatile and easily decomposed at higher temperatures (Kiss *et al.*, 2000).

Experimental

Callus induction

Stem explants of *E. cinerascens* were cultured in Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 0.5 mg/L 6-benzylaminopurine and α-naphthaleneacetic acid in combination. The cultures were kept in the culture room and maintained at 25 ± 2°C with 16 hours light/8 hours dark photoperiod. After a few days, light green callus were produced and subsequently subcultured for several times before the callus were harvested for extraction.

Preparation of callus extracts

Extraction was performed in dim or subdued light to avoid pigment degradation. Initially, fresh samples of callus were cut into small pieces. After that, 0.1 g of the callus was weighed and extracted separately by using five different types and concentrations of organic solvents, namely, 100 per cent acetone, 80 per cent acetone, 95 per cent ethanol, 100 per cent methanol and 90 per cent methanol. A small amount of $MgCO_3$ powder was added while grinding the samples to neutralise plant acids and to prevent conversion of Chl a to phaeophytin a (Lichtenthaler and Buschmann, 2001a). Besides, a small amount of sand was also added to assist in cell lyses and complete the extraction of samples. Homogenated samples were then centrifuged at 5,000 rpm for 10 min at 24°C to get a clear supernatant. The supernatant consisting pigments of interest was pipetted into a clean vessel, which was wrapped with aluminium foil and maintained under dark condition (Lichtenthaler and Buschmann, 2001a) before subjected to spectroscopic and chromatographic analysis as the pigments are light and heat sensitive.

Spectroscopic analysis

The supernatant was transferred into 3.0 mL cuvette, and spectrophotometric measurements were made directly via UV-1650 PC UV-V_{IS} Spectrophotometer (Shimadzu). Absorbance spectra were monitored in range of 300-800 nm wavelength. The pigments were identified through the absorbance reading and quantified using "Spectroscopic quantification equation" derived from Lambert–Beer law (Lichtenthaler and Buschmann, 2001b; Wellburn, 1994). Results were subjected to one-way analysis of variance (ANOVA) and statistically analysed by Duncan's multiple range test (DMRT). Means listed in the tables with different letters in the same column are significantly different at $p = 0.01$.

Chromatographic analysis

The solvent (in liquid form) was evaporated in a rotary evaporator (BUCHI Rotavapor R-114) and the residue (extract) was left in powder form, which was later dissolved in polar (methanol and acetone) and non-polar (petroleum ether) solvents. In this study, TLC was carried out using silica gel 60 F254 chromatography plate. Once the pigment separation was completed, the plate was placed under visible and UV254 light to observe the spots which formed on the plate, and Rf

(retention factor) values were calculated afterward. The procedures were repeated few times using different ratios of solvent systems or mobile phases (a mixture of organic solvents – petroleum ether:chlorophyll:ethyl acetate) to obtain good pigment separation. Initially, the extract was spotted on the plate using microcapillary tube before placing it in the TLC chamber containing different ratios of solvent systems that have been developed, such as 35:8:1, 26:3:1, 25:2:3 and 20:8:2. The solvent systems took a few minutes to move up, and the plate was removed as the solvent reached solvent front line. As the solvent slowly travelled through the plate, the pigments were separated in the spots form, which could be observed under visible and UV light. Rf values were calculated to identify pigments extracted from callus of the species; Rf value is a distance travelled by the pigment divided by the distance travelled by the solvent.

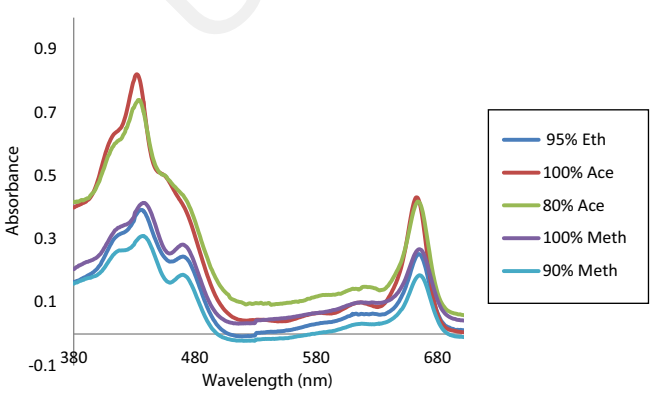
Results and discussion

Ultraviolet-visible absorbance

The experiment was carried out in dim or subdued light as recommended in previous research (Melendez-Martinez *et al.*, 2007; Boyer, 1990). It is advisable to proceed with pigment detection immediately after pigment extraction to avoid evaporation of solvents and pigment degradation. In this experiment, fresh samples were preferred instead of dried samples as the absorption in the red and blue maxima is the highest in freshly isolated pigments. Nevertheless, a previous study has reported that fresh samples are high in water content. Thus, the samples were suggested to be freeze-dried before extracted and analysed using a spectroscopic method (Lichtenthaler and Buschmann, 2001a).

By UV-Vis spectrophotometer, the intensity of pigment was depicted through visible spectrum. In fact, the absorption maxima of chlorophylls were observed in two regions, blue region near 412 and 436 nm wavelength and red region near 642 and 665 nm wavelength. Conversely, the absorption maxima of carotenoids were only discovered in blue region with three shoulders between 400 and 500 nm wavelength. Generally, different types of pigments present different shape of spectra and peak because each peak is unique to one pigment for a particular solvent. Figure 1 shows almost similar shape of

Figure 1 Absorption spectra of pigments extracted from fresh callus of *E. cinerascens* in various solvent extractions



spectra for pigments extracted from fresh callus in various solvent extractions. It was found that the shape of spectrum for 100 and 80 per cent Acetone is little bit different from 100 per cent methanol, 90 per cent methanol and 95 per cent ethanol. Next, 100 per cent acetone exhibited the highest absorbance reading in both blue and red region, followed by 80 per cent acetone, 100 per cent methanol, 95 per cent ethanol and 90 per cent methanol. Analysis of diverse solvent polarities on pigment extraction has proven that the absorption maxima of pigment shifted to longer wavelength as the polarity of solvent increased. This finding is comparable with a previous study which indicated that increase in polarity led to a shift in the absorption maxima from 660 to 665 nm and from 428 to 432 nm for Chl a, and from 642 to 652 nm and from 452 to 469 nm for Chl b (Lichtenthaler and Buschmann, 2001b). Furthermore, the absorption maxima also shifted to longer wavelength due to the present of high water content in the sample (Boyer, 1990). Hence, the present study verified that the absorptions maxima rely on solvent extraction polarity and the type of sample used, which is concurrent with previous reports.

The shape of spectra provides important characteristic for identification of purified pigments (Scott, 2001). As noted, earlier studies have stated that absorption maxima of Chl a and b were 661.6 and 644.8 nm in 100 per cent acetone, 664.2 and 648.6 nm in 95 per cent ethanol and 665.2 and 652.4 nm in 100 per cent methanol, respectively (Lichtenthaler and Buschmann, 2001b). Meanwhile, the absorption maxima detected for carotenoids was 470 nm (Lichtenthaler and Buschmann, 2001a). Based on the evidence, the pigments extracted from the callus were measured, and a comparison of pigment content, including chlorophyll a, chlorophyll b, total chlorophylls, total carotenoids and total pigment content, in different solvent extractions are recorded (Table I). Interestingly, 80 per cent acetone showed the highest chlorophyll a and chlorophyll b content, with 22.61 and 11.10 mg/g, respectively whereas 90 per cent methanol showed the lowest chlorophyll a and chlorophyll b content, which corresponds to 11.42 and 1.23 mg/g. Therefore, the highest total of chlorophyll content, 33.71 mg/g, was observed in 80 per cent acetone, while the lowest total of chlorophyll content, 12.65 mg/g, was observed in 90 per cent methanol. Nevertheless, the highest total of carotenoid content, 7.40 mg/g, was obtained in 100 per cent acetone, whilst the lowest total of carotenoid content, 2.72 mg/g, was obtained in 100 per cent methanol. Overall, the highest total of pigment content was 39.84 mg/g, which was observed in 80 per cent acetone, followed by 100 per cent acetone, 100 per cent methanol, 95 per cent ethanol and 90 per cent methanol, which correspond to 36.33, 25.09, 22.00 and 16.18 mg/g, respectively. As a result, 80 per cent acetone was identified as the most efficient solvent for pigment extraction of callus of *E. cinerascens*, which contradicts with former research that promoted dimethylformamide was the efficient solvent (Moran and Porath, 1980).

Conversely, analysis of the ratio of pigment content extracted from callus in different solvent extractions (Table II) indicated that the highest ratio of chlorophyll a to chlorophyll b of 9.28 was obtained in 90 per cent methanol. However, the lowest ratio of chlorophyll a to chlorophyll b of 2.04 was obtained in 80 per cent acetone. In contrast, the highest and lowest ratios of

Table I Comparison of chlorophylls, carotenoids and total of pigment content (mg/g fresh weight) from callus of *E. cinerascens* measured in different solvent extractions

Solvent extraction	Pigment content (mg/g) Ca	Cb	C(a + b)	C(x + c)	Total
100% acetone	$22.34 \pm 0.04_b$	$6.59 \pm 0.05_c$	$28.93 \pm 0.02_b$	$7.40 \pm 0.02_a$	$36.33 \pm 0.02_b$
80% acetone	$22.61 \pm 0.05_a$	$11.10 \pm 0.01_a$	$33.71 \pm 0.01_a$	$6.13 \pm 0.04_b$	$39.84 \pm 0.05_a$
95% ethanol	$14.12 \pm 0.05_d$	$4.07 \pm 0.03_d$	$18.19 \pm 0.06_d$	$3.81 \pm 0.01_c$	$22.00 \pm 0.08_d$
100% methanol	$15.05 \pm 0.02_c$	$6.85 \pm 0.03_b$	$21.90 \pm 0.02_c$	$3.03 \pm 0.07_e$	$24.93 \pm 0.02_c$
90% methanol	$11.42 \pm 0.05_e$	$1.23 \pm 0.05_e$	$12.65 \pm 0.03_e$	$3.53 \pm 0.02_d$	$16.18 \pm 0.01_e$

Notes: Data represent mean value ± standard error (SE) for three replicates of each treatment. Means with different letters in the same column are significantly different at $p = 0.01$ according to DMRT. Ca: chlorophyll a; Cb: chlorophyll b, C(a + b): total chlorophyll a and b; and C(x + c): total carotenoid (xanthophyll and carotene); subscripts a, b, c, d and e are significant to determine the best solvent to extract pigments (total), carotenoids [C(x + c)], chlorophylls [C(a + b)] or chlorophyll a (Ca) and chlorophyll b (Cb) specifically

Table II Ratio of pigments content (mg/g fresh weight) extracted from callus of *E. cinerascens* in different solvent extractions

Solvent extraction	Pigment content (mg/g) Ca/Cb ratio	C(a + b)/C(x + c) ratio
100% acetone	$3.39 \pm 0.02_b$	$3.91 \pm 0.01_d$
80% acetone	$2.04 \pm 0.02_d$	$5.50 \pm 0.01_b$
95% ethanol	$3.47 \pm 0.03_b$	$4.77 \pm 0.03_c$
100% methanol	$2.20 \pm 0.01_c$	$7.23 \pm 0.02_a$
90% methanol	$9.28 \pm 0.05_a$	$3.58 \pm 0.04_e$

Notes: Data represent mean value ± standard error (SE) for three replicates of each treatment. Means with different letters in the same column are significantly different at $p = 0.01$ according to DMRT. Ca: chlorophyll a; Cb: chlorophyll b; C(a + b): total chlorophyll a and b; and C(x + c): total carotenoid (xanthophyll and carotene); subscripts a, b, c, d and e are significant to promote the sample condition. As stated in the manuscript, ratio Ca/Cb indicates the functional pigment equipment and light adaptation of photosynthetic apparatus while ratio C(a + b)/C(x + c) indicates the greenness of plants (normal, senescence, stress or damage)

chlorophylls to carotenoids were obtained in 100 and 90 per cent methanol, with ratio of 7.23 and 3.58, respectively.

Ratio Ca/Cb is very important as the indicator of functional pigment equipment and light adaptation of photosynthetic apparatus, while ratio C(a + b)/C(x + c) is an indicator of the greenness of plants (Lichtenthaler and Buschmann, 2001b). According to the analysis, results verified that the callus samples possess a normal ratio (4.2-5.0), comparable to most sun leaves and sun-exposed plants, while others with a lower ratio (≤3.5) may promote the occurrence of senescence, stress and damage to the plant or photosynthetic apparatus.

Thin layer chromatography

TLC was conducted to separate chemically similar substances. Once the pigments completely separated, a chromatogram formed and Rf values were calculated. Photography of chromatograms is an applicable practice for recording and preserving TLC analysis results (Sherma, 2000). Figure 2 illustrates chromatograms of pigment separation in methanol (M), acetone (A) and petroleum ether (P) extracts with different solvent systems (f, g, h, i) applied (Figure 2). In general, the chromatograms promoted various spots in different colours (yellow, blue, green and orange) as viewed under UV light, and the results clarified that petroleum ether extract promoted more spots compared to methanol and acetone extracts.

Rf values for each of the spots were calculated and are presented in Table III. Analysis of the results demonstrated good pigment separation in solvent system h (25:2:3) for acetone extract, but better pigment separation was detected in solvent system i (20:8:2) for methanol extract. Nevertheless, the best pigment separation was obtained in solvent system g (26:3:1) for petroleum ether extract with four spots, which indicated that the callus contained carotene, phaeophytin, chlorophyll a and xanthophyll. Indeed, Rf value of carotene was in range of 0.96-0.98, phaeophytin in the range of 0.70-0.81, chlorophyll a in the range of 0.58-0.59, chlorophyll b in the range of 0.42-0.48 and xanthophyll in the range of 0.15-0.35. Phaeophytin is a degraded by-product of chlorophyll (Katayama *et al.*, 2003).

Basically, Rf value varied with different solvent system and chromatography material used. Very small changes in the composition of solvent system resulted in different Rf values. A previous report disclosed that Rf value decreased with an increase in the concentration of organic component in the mobile phase (Kiss *et al.*, 2000). Two factors affected the distance of pigment travel, namely, the solubility of the pigment and the absorption of pigment onto the TLC plate. More soluble pigment travels greater distance and travels slower as more pigments absorb onto the plate.

Both of the methods (spectrophotometric and chromatographic) permit the identification of pigments (Criado *et al.*, 2007) but through different approaches. By spectroscopy, pigments were identified through the absorption spectra illustrated in the specific wavelength, whereas by chromatography, pigments were separated and depicted in a spot form on the chromatogram plate. Practically, spectrophotometry is usually carried out as complementary to chromatographic analysis (Melendez-Martinez *et al.*, 2007).

Conclusions

Two types of pigments have been identified in the callus of *E. cinerascens*, namely, tetraphyrroles (chlorophyll *a* and *b*) and carotenoids (carotene and xanthophyll). Both pigments were detected and quantified via spectroscopic and chromatographic

Figure 2 Chromatogram of pigments separation in methanol (M), acetone (A) and petroleum ether (P) extracts

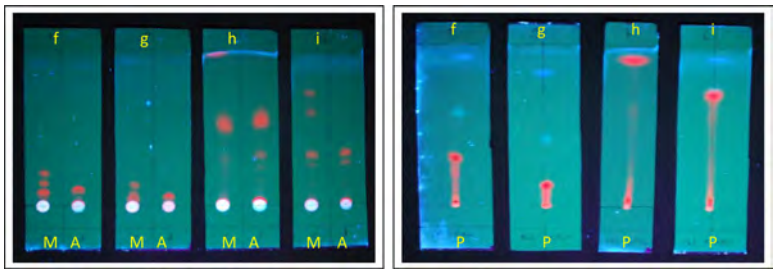


Table III Rf values of pigments in various extracts (methanol, acetone and petroleum ether) with different solvent systems (f, g, h and i) applied

Extract	Solvent system			
	f (35:8:1)	g (26:3:1)	h (25:2:3)	i (20:8:2)
Methanol (M)	0.10	0.13	0.54	0.36
	0.17		0.99	0.70
	0.23			0.83
Acetone (A)	0.11	0.09	0.29	0.29
			0.57	0.36
Petroleum ether (P)	0.30	0.14	0.93	0.74
	0.63	0.43	0.99	0.93
	0.93	0.83	1.00	0.97
	1.00	1.00		

methods. The highest total of pigment content was observed in 80 per cent acetone with 39.84 mg/g. Therefore, 80 per cent acetone was the most efficient solvent for pigment extraction of callus of *E. cinerascens*. Besides, it can be concluded that pigment extraction was influenced by types of solvent extractions, samples, equipment and methods used. Further research should be done using the most frequent and powerful separation methods such as reverse-phase TLC or HPLC and mass spectroscopy to verify and determine pigments content in the callus of *E. cinerascens* and to study more details regarding its beneficial and potential application in the future.

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The effect of zeatin and gibberellic acid on regeneration and in vitro flowering of Phlox paniculata L.

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Technical Note

Effects of zeatin and gibberellic acid on regeneration and in vitro flowering of Phlox paniculata L.

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Abstract : The present work reports on the regeneration and in vitro flowering of Phlox paniculata L. Different explants (leaves, petioles and stems) were cultured on Murashige and Skoog (MS) solid medium supplemented with different concentrations and combinations of α-napthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP). Among the explants studied, leaves were found to be the most responsive and had the highest regeneration frequency (100%) when they were cultured in the presence of 8.0 µM NAA and 6.6 µM BAP. However, the regenerated plantlets did not produce any flower buds in successive subcultures. In vitro flowering was only induced after 4 weeks of culturing from shoot tip segments which were maintained on MS solid medium supplemented with zeatin (2.2-11.2 µM) or gibberellic acid (GA_3,1.4-7.2 µM). In the present study treatment with 11.2 µM zeatin showed the highest frequency of in vitro flowering (67.8 ±3.2%), whereas 5.6 µM GA_3 gave a lower percentage (48.8±9.3%). Although the flowers generated in vitro were sterile, our study would provide an important step towards future investigation on the essential factors in in vitro flowering in P. paniculata and to elucidate other developmental, physiological and environmental stimuli, which are required for promoting or inhibiting the transition of a vegetative state to a flowering state in this species.

Keywords: Phlox paniculata L., in vitro flowering, zeatin, gibberellic acid, micropropagation

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INTRODUCTION

Phlox paniculata L. is an important flowering plant that belongs to Polemoniaceae [1]. P. paniculata L. is a perennial ornamental plant, bears dense terminal clusters of flowers in pink, crimson and mauve, is commonly grown as borders, and is also suitable for window boxes and tubs [2-4]. The species produces sterile seeds [1, 2], and even though the traditional method of propagation is by root cuttings, the roots are often damaged by red ants and sometimes by soil-born fungi. Therefore, in vitro culture is an effective technique for obtaining large-scale clonal propagation of P. paniculata. Schnabelrauch and Sink [5] studied the clonal propagation of P. paniculata through axillary bud culture. Later, shoot regeneration was induced from adult leaf segments cultured by Declerck and Korban [6]. These studies revealed the potential for inducing multiple shoots in in vitro cultures of this species. Moreover, multiple shoot regeneration and the in vitro technique are more advantageous for rapidly obtaining clonal plants as well as for conservation [7-11]. In some studies rapid micropropagation of plants is achieved through synthetic seed technology and somatic embryos [12-16]. Besides in vitro flowering, valuable bioactive molecules that promote antidiabetic activity, antioxidation and anticancer activity [17-21], as well as provide pigments for paint and coating technology are also obtained from plants either grown in vivo or in vitro [22-28].

Flowering is a unique developmental event in plants, which involves a transition from the vegetative shoot apex to either an inflorescence or a floral meristem, followed by initiation and subsequent maturation of the floral organ [29]. The flowering process is one of the most critical stages in plant life and is vital for the completion of the life cycle and seed production. Under natural growth, the flower formation usually begins when a plant reaches maturity. The transition from the vegetative state to the floral stage is considered to be a complex process regulated by a combination of various environmental and genetic factors. Some of the important factors are plant growth regulators, carbohydrates, light and pH of the culture medium [30]. However, the mechanism of the transition from the vegetative to reproductive state is not well understood in most plants. In vitro flowering is an important tool for minimising the influence of environmental factors and therefore this technique clarifies the key influences on flowering by a precise control of plant growth regulators. Furthermore, in vitro flowering also provides an ideal experimental system for plants grown in vivo in order to study the biological mechanism of flowering. In the present work influences of plant growth regulators on clonal propagation with different explants and in vitro flowering from shoot apices of P. paniculata have been examined. As far as we know, in vitro flowering in 'Garden Phlox' has not been reported. In the current work we used various concentrations of α-napthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) to simultaneously induce direct regeneration and in vitro flowering from shoot tips and other explants of this species using zeatin and gibberellic acid (GA_3).

MATERIALS AND METHODS

Plant Materials and Culture Conditions for in Vitro Shoot Regeneration

Three types of explants from leaves, petioles and stems were collected from intact garden-raised mature plants during their vegetative stages. All explants were initially washed with tap water for 30 min., surface-sterilised with 50% commercial bleach (containing 5% sodium hypochlorite) for 1 min., then rinsed with sterile distilled water at least three times and finally dipped in 70% (v/v) ethanol for 1 min., followed by rinsing with sterile distilled water three times. These sterile explants

were cut into segments (approx. 5-10 mm in length for both petioles and stems and around 5x5-mm^2 pieces for leaves) and cultured on Murashige and Skoog solid medium [31] (0.8% Agar Technical, No.3, Oxoid Ltd., England) supplemented without (control) or with combinations of 2.6, 5.3, 8.0 and 10.6 µM NAA (Sigma-Aldrich, USA) and 2.2, 4.4, 6.6 and 8.8 µM BAP (Sigma-Aldrich, USA). The pH of the media was adjusted to 5.8 and they were then sterilised by autoclaving at 121ºC and 103 kpa for 20 min. All the cultures were maintained at 25 ± 1ºC with a 16-h photoperiod (1000 lux).

Explant Preparation and Culture Conditions for in Vitro Flowering

Shoot apices were collected from 4-month-old axenic cultures (vegetative stage) of the P. paniculata plant. The explants were further aseptically excised into small pieces containing meristems with 3-4 true leaf primordia and cultured on the solid MS medium supplemented without (control) or with various concentrations (2.2, 4.5, 6.7, 9.0 and 11.2 µM) of zeatin (Sigma-Aldrich, USA) or GA_3 (Sigma-Aldrich, USA) at 1.4, 2.8, 4.3, 5.6 and 7.2 µM. The pH of the media was adjusted to 5.8 and they were then sterilised by autoclaving (121ºC, 103 kpa) for 20 min. All the cultures were incubated at 25 ± 1 ºC with a 16-h photoperiod (1000 lux).

Morphological Analysis

All cultures were continuously observed from one week of treatment to evaluate their development by counting the total number of shoot buds/leaves initiated by the explants, and the state of the apical meristem, either vegetative or floral, was recorded. A meristem was classified as floral when the first sepal primordium of the flower that characterises the reproductive structure was visible. It was referred to as 'vegetative' when there was no apparent reproductive morphogenesis. The presence or absence of a basal callus and roots was also noted. Each experiment was repeated at least twice with 10 explants per treatment. Pooled results of the different experiments were analysed and presented.

Statistical Analysis

All data and variables were statistically analysed using SPSS statistical package version 11. Values were presented as mean ±SE. One-way ANOVA and Multiple Range Analysis were done on all data using 95% LSD intervals method.

RESULTS AND DISCUSSION

Shoot Regeneration from Different Explants

Initially, three types of explants were obtained from mature plants; leaves, petioles and stems were sterilised and cultured on MS solid medium supplemented without (control) or with different levels of phytohormones as described in the Materials and Methods section. In the beginning of the culture direct shoot bud formation was observed in all types of explants when treated with phytohormones (NAA and BAP) but not in the control. However, none of the explants showed callus formation. Within 5 weeks of culture, vegetative buds further developed and regenerated into shoots (Figure 1a). The highest frequency (100%) of shoot regeneration was obtained from leaf explants when cultured on medium supplemented with 8.0 µM NAA and 6.6 µM BAP (Figure 1b). However, at this level of phytohormones, maximum shoot regeneration was also observed in petiole and stem explants with a rate of 67.8±3.2 and 48.8±9.3% respectively (Figure 1b). Subsequently,

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root formation was observed in all clonal shoots within 11 weeks and they all developed into individual plantlets. When acclimatised and transferred to the field, about 90% of the clonal plantlets survived under natural environmental conditions without showing any morphological variation during their development.

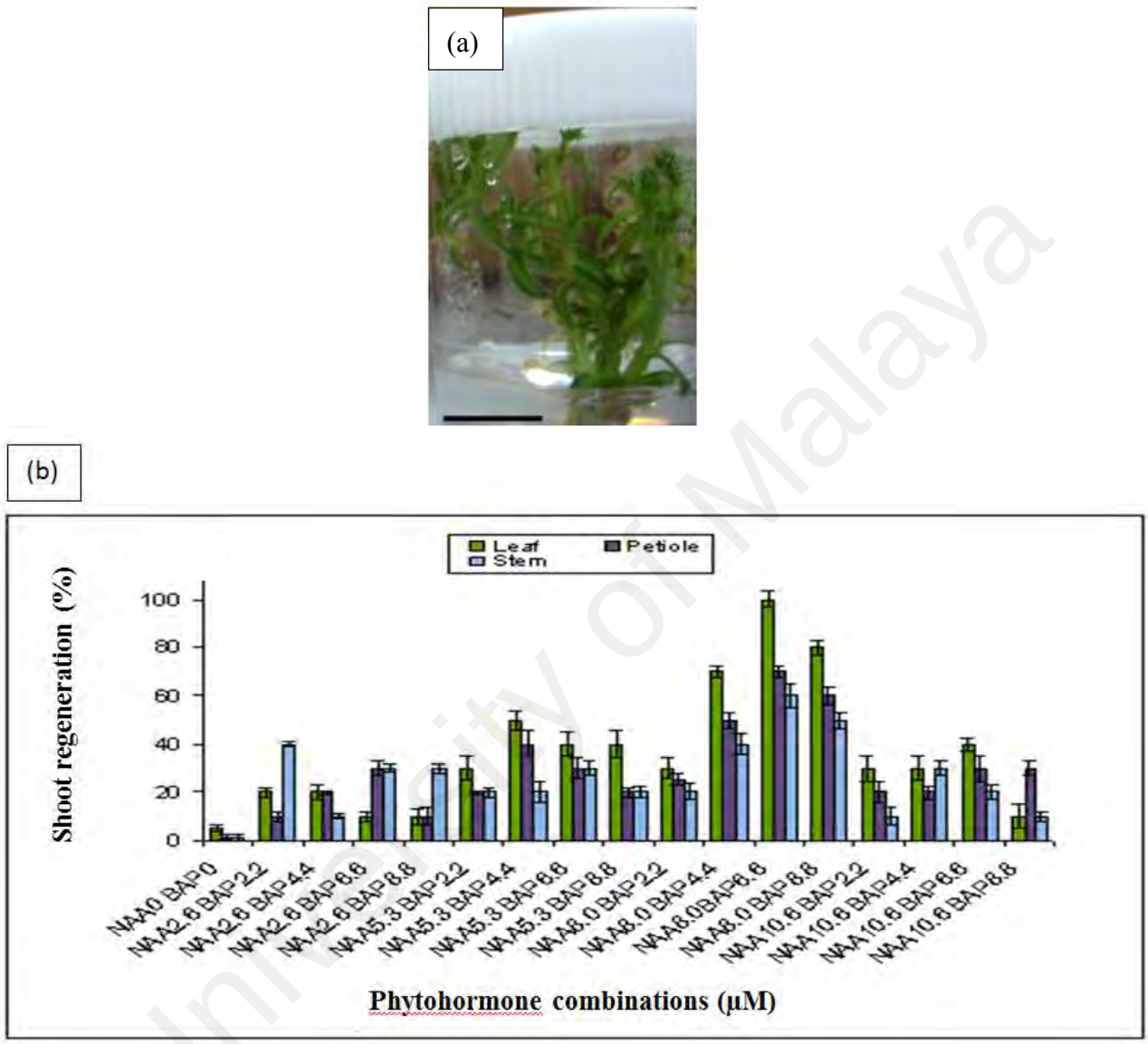

Figure 1. In vitro shoot regeneration of Phlox paniculata: (a) profuse vegetative shoot formation from mature leaf explants cultured on MS solid medium supplemented with NAA and BAP (8.0 and 6.6 µM respectively) in plastic sterile tubes after 5 weeks. The bar indicates 1 cm; (b) graph showing in vitro shoot regeneration pattern from different explants in response to various concentrations of NAA and BAP, after 8 weeks of culture

Cytokinin and auxin addition for the in vitro shoot regeneration has been studied in many plant species [32-34]. Various reports have shown that the in vitro shoot regeneration can be successfully induced by using a combination of BAP and NAA [35-38]. In the medicinal plant Withania somnifera cultured on MS medium supplemented with 8.8 µM BAP and 0.5 µM NAA, multiple shoot regeneration was observed [39]. This was also observed in Celosia argentea cultured on MS medium supplemented with 1.0 mg/L BAP and 0.5 or 1.0 mg/L NAA [40]. In addition, in an in

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vitro multiple shoot regeneration protocol of Boerhaavia diffusa by Roy [41], it was shown that a maximum frequency of 90% could be obtained only when it was cultured in MS medium containing BAP (6.6 μM) and NAA (2.6 μM). Consistent with those previous findings, in the present work we have also shown that NAA (8.0 μM) plus BAP (6.6 μM) treatment generates a maximum frequency of multiple shoot regeneration in vitro in P. paniculata. Even though NAA promotes rooting, there is a synergistic effect in combination with a slightly higher NAA concentration for inducing multiple shoots in P. paniculata. Similar findings were also reported by Elias et al. [42] and Ahmed et al. [43] in the in vitro shoot formation of Echinocereus cinerascens and Phyla nodiflora respectively. Thus, the success in raising plants through direct regeneration and bypassing the callogenesis phase has opened up the possibility for a large-scale clonal propagation of P. paniculata. When other combinations of phytohormones, like kinetin and NAA, kinetin and 3-indole acetic acid (IAA), BAP and 2,4-dichlorophenoxyacetic acid (2,4-D) or kinetin and 2,4-D, were used at different concentrations, profuse shoot regeneration was not evident in all three types of cultured segments. However, in the current work successive subculturing of the regenerated plants did not show further morphogenetic differentiation such as floral transition.

In Vitro Flowering

To induce in vitro flowering, shoot tip explants were further collected from 4-month-old aseptic plants and cultured on MS solid medium supplemented with various concentrations of singly applied zeatin and GA_3 (Table 1). After 4 weeks of treatment, the development of multiple shoots and initiation of flower buds were observed from cultured explants only when treated with NAA or BAP but not in the control (Table 1). Their successive subcultures generated white flowers with purple or pink stripes (Figure 2). In this experiment 3-4 flowers per plantlet developed within 7-8 weeks of culture. Treatment with zeatin at a concentration of 11.2 μM resulted in the highest frequency (67.8±3.2%), whereas 5.6 μM GA_3 gave 48.8±9.3% of in vitro flower induction (Table 1). However, treatment with either zeatin or GA_3 resulted in the formation of white or pink flowers occasionally. All the developed flowers were approximately 1.7-2.0 cm in width and were formed from the apical and axillary buds (Figure 2). Each bloom had 5 sepals, slightly extended and pointed at their middles with an extended and fused throat that opens into 5 distinct and overlapping lobes. However, the in vitro flowers failed to develop other reproductive organs such as stamens, stigmas or pistils even when they were subcultured for a long period of about 10 months

In earlier studies the cytokinins requirement for the growth and development of flower buds has been reported in both monocots [44] and dicots [45-46]. The promotion of in vitro flowering by cytokinins was repeatedly reported [47-53]. The influence of cytokinins on the in vitro flowering of Perilla frutescens is surprising [54]. Also, the beneficial effect of cytokinins on the induction of flowering for other plants was reported in orchids [55], Fortunella hindsii [56] and Lemna [57]. The results presented here in Table 1 are similar to those previous findings. However, high concentrations of zeatin caused inhibition of in vitro flowering (data not shown).

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Table 1. In vitro flowering of Phlox paniculata on MS medium supplemented with different concentrations of zeatin and GA_3

Treatment (μM)	Response	Mean % of flowering plantlets (±SE)
MS + zeatin (0.0)	No flowering response	0
MS + zeatin (2.2)	Flowering response	21.6 ± 3.7 de
MS + zeatin (4.5)	Flowering response	25.6 ± 2.9 d
MS + zeatin (6.7)	Flowering response	26.9 ± 2.8 d
MS + zeatin (9.0)	Flowering response	37.0 ± 3.3 c
MS + zeatin (11.2)	Flowering response	67.8 ± 3.2 a
MS + GA_3 (0.0)	No flowering response	0
MS + GA_3 (1.4)	Flowering response	35.1 ± 8.4 c
MS + GA_3 (2.8)	Flowering response	39.0 ± 3.6 bc
MS + GA_3 (4.3)	Flowering response	47.9 ± 3.8 b
MS + GA_3 (5.6)	Flowering response	48.8 ± 9.3 b
MS + GA_3 (7.2)	Flowering response	34.4 ± 2.3 cd

Note: Mean values followed by the same letter within a column are not significantly different at the 0.05 level according to LSD test.

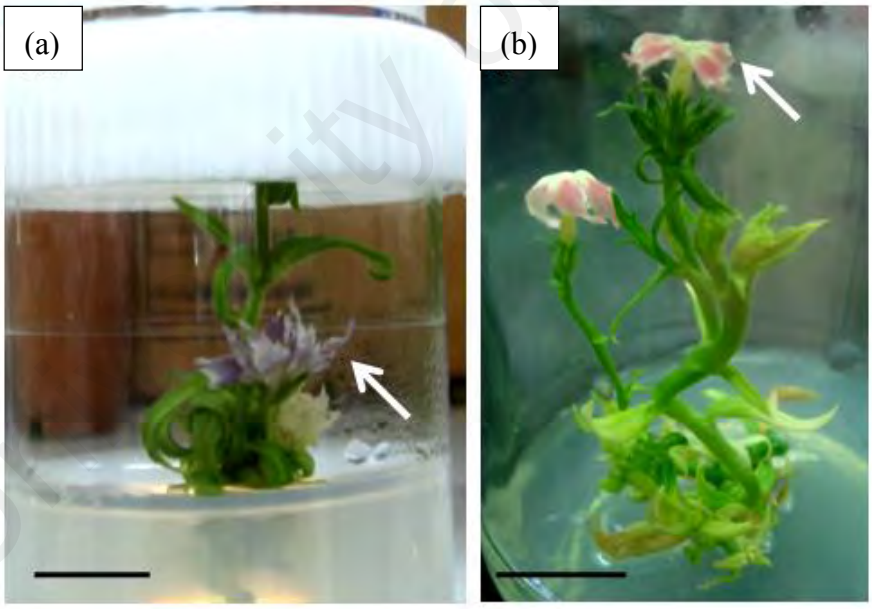

Figure 2. Flower development after 6 weeks on shoot tip explant culture of Phlox paniculata. The MS medium, supplemented with zeatin (11.2 μM) or GA_3 (5.6 μM), gives shoot multiplication with flowers (arrowed) of purple (a) or pink stripes (b). Bar indicates 1 cm.

In certain plants auxin has been reported to be either ineffective or inhibitory [58] in the in vitro flowering induction process. In this study an absence of in vitro flowering formation was observed when different concentrations of 2,4-D and IAA were utilised in the culture medium. In contrast, gibberellins have been reported as an inducer of the flowering process in several long-day and cold-requiring rosette plants [59]. GA_3 was even able to regenerate sporophytes from gametophyte

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explants in ferns [60]. In earlier work it has been postulated that a GA_3 promotive pathway exists in Arabidopsis thaliana [61], where GA_3 activates the LEAFY (a floral meristem identity gene) transcription [62]. Considering these findings, our results support the idea that independently, zeatin or GA_3 may form one of the key factors without which floral bud initiation and their subsequent development are not possible in P. paniculata. However, a detailed study on the actual mode of action of zeatin and GA_3 in the in vitro flowering process in Phlox remains to be examined. In both cases, two to four flowers were produced from each in vitro cultured explant (Figure 2). However, neither the flowers supplemented with zeatin nor with GA_3 showed fruiting in the subsequent culture. This might have been due to the absence of other reproductive organs. In the current study complete flowering was not observed even when they were cultured in the presence of kinetin and IAA; perhaps different conditions might be required for the induction and development of normal flowers. In previous study by Taha [63] on Murraya paniculata, complete plant regeneration was achieved from portions of cotyledons and shoot explants when they were cultured in MS medium supplemented with 4.44 μM BAP. On subsequent subculture on MS basal medium, 80% of flowering was obtained, while MS medium fortified with 2.69-10.74 μM NAA gave 62-72% of flowering [63]. In Begonia x hiemalis, the best explant for in vitro flowering was inflorescence cultured on MS medium supplemented with 4.44 μM BAP, 5.37 μM NAA, 4% sucrose and 40 mg/L adenine [64]. It seems that different species require different hormonal regimes for in vitro flowering.

Flowering is an important phase in the developmental processes of floricultural crops. In this study an attempt was made to identify the most favourable set of environmental and nutritional conditions for adventitious shoot regeneration and flower induction in vitro. An interesting feature of the present study is that the process of in vitro shoot apex flowering is responsive to only zeatin or GA_3. This is a significant phenomenon considering the fact that the explants were obtained from axenic cultures and that it is possible to avoid the maturation period spanning several months before a plant produces flowers. Further experiments should lead to a better understanding of the physiological and molecular events underlying the shift from the vegetative state to the flowering state, as well as a better understanding of factors related to overcoming flower sterility and of seed formation in vitro.

CONCLUSIONS

Among the explants tested, those from leaf segments were found to be the most responsive and have the highest regeneration frequency (100%) when cultured in the presence of 8.0 μM NAA and 6.6 μM BAP, whereas treatment with 11.2 μM zeatin gave the highest frequency of in vitro flowering (67.8 ±3.2%). This protocol may be extended to plant breeding studies for the purpose of quick flowering and fruit and seed formation under in vitro conditions.

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