COMPARATIVE STUDIES ON TISSUE CULTURE, MORPHOLOGICAL AND ANTIMICROBIAL PROPERTIES OF *Clitoria ternatea* L. AND *Onobrychis viciifolia* Scop.

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ABSTRACT

In the present study, tissue culture technique was used for *Clitoria ternatea* L. (tropical legume) and *Onobrychis viciifolia* Scop. (temperate legume) for comparison in plant regeneration by manipulation and exploitation of different temperatures (18, 24 and 30±1ºC), different concentrations of adenine hemisulfate ($ADSO_4$), auxins (NAA and 2,4-D), cytokinins (BAP and KIN) and different explant sources (root, stem and flower bud). The best temperature for seeds germination for both species was at 24±1ºC, grown *in vivo* and *in vitro*. The optimum regeneration medium for *C. ternatea* L. was MS supplemented with 40.0 mg/L $ADSO_4$, 2.0 mg/L NAA and 1.0 mg/L BAP which resulted in shoots (12.03±0.12) and roots (8.72±1.27) formation, whilst, the optimum medium for *O. viciifolia* Scop. was MS supplemented with 40.0 mg/L $ADSO_4$, 1.0 mg/L 2,4-D and 3.0 mg/L KIN with formation of shoots (17.97±0.09) and roots (4.11±0.42). The optimum medium for synthetic seed production was obtained on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$, 2.0 mg/L NAA and 1.0 mg/L BAP for *C. ternatea* L., while *O. viciifolia* Scop. on MS medium supplemented with 40.0 mg/L $ADSO_4$, 1.0 mg/L NAA and 2.0 mg/L BAP, by using 3.0% (w/v) sodium alginate ($NaC_6H_7O_6$) and soaked in 100 mM (w/v) calcium chloride dehydrate ($CaCl_2.2H_2O$) solution for 30 minutes. Synthetic seed production was initiated from embryogenic callus of both species and the seeds managed to survive for 3 months. All plantlets obtained were successfully acclimatized with 91.07±0.96% survival rate for *C. ternatea* L. and 71.58±1.27% survival rate for *O. viciifolia* Scop. Due to the importance of micromorphological features for the taxonomic consideration, FESEM and histology analysis revealed the presence of shallow pit of stomata and non-glandular trichomes with sharp-point end, the same as mother plant indicating no occurance of somaclonal variations. These species responded positively against bacteria *(Bacillus subtilis,*

Staphylococcus aureus and Escherichia coli) and fungi *(Fusarium* sp., *Candida albican* and *Trichoderma* sp.). For potential use in paint technology, it was found that anthocyanin from vivid blue petals of *C. ternatea* L., was able to withstand pH, heat, UV and NaCl. Generally, the anthocyanin absorbance values of *C. ternatea* L. decreased when tested with higher pH, longer duration of UV radiation, higher concentrations of NaCl and longer exposure of heat (at 50±1ºC). The colour of this anthocyanin influenced by pH (red at pH 1, purple at pH 4.5, blue at pH 5.5 and cyan at pH 6.5) and became darker in the oven (at 50±1ºC) as the duration increased. The additional of 1% tartaric acid to anthocyanin-PMMA was found to be the best stabilizer to improve the colorant stability and durability. Overall, *C. ternatea* L. capable to adapt in both tropical and temperate regions without any geographical and seasonal constraints for year round commercial cultivation.

ABSTRAK

Dalam kajian ini, teknik kultur tisu telah digunakan untuk *Clitoria ternatea* L. (kekacang beriklim tropika) dan *Onobrychis viciifolia* Scop. (kekacang beriklim sederhana) untuk perbandingan dalam pertumbuhan semula tumbuhan melalui manipulasi dan eksploitasi suhu yang berbeza (18, 24 dan 30 ± 1ºC), kepekatan adenine hemisulfate ($ADSO_4$) yang berbeza, auksin (NAA dan 2,4-D), sitokinin (BAP dan KIN) dan sumber eksplan yang berbeza (akar, batang dan putik bunga). Suhu terbaik untuk percambahan biji benih untuk kedua-dua spesies adalah pada 24±1ºC, yang ditanam *in vivo* dan *in vitro*. Medium yang optimum untuk pertumbuhan semula *C. ternatea* L. adalah MS ditambah dengan 40.0 mg/L $ADSO_4$, 2.0 mg/L NAA dan 1.0 mg/L BAP yang mengakibatkan pembentukan pucuk (12.03±0.12) dan akar (8.72±1.27), manakala, medium optimum bagi *O. viciifolia* Scop. adalah MS ditambah dengan 40.0 mg/L $ADSO_4$, 1.0 mg/L 2,4-D dan 3.0 mg/L KIN dengan pembentukan pucuk (17.97±0.09) dan akar (4.11±0.42). Medium optimum untuk pengeluaran benih sintetik telah diperolehi pada MS ditambah dengan kombinasi 40.0 mg/L $ADSO_4$, 2.0 mg/L NAA dan 1.0 mg/L BAP untuk *C. ternatea* L., manakala *O. viciifolia* Scop., MS ditambah dengan 40.0 mg/L $ADSO_4$, 1.0 mg/L NAA dan 2.0 mg/L BAP, dengan menggunakan 3.0% (w/v) natrium alginate ($NaC_6H_7O_6$) dan direndam dalam 100 mM (w/v) kalsium klorida mendehidrasi ($CaCl_2.2H_2O$) selama 30 minit. Pengeluaran benih sintetik telah dimulakan daripada kalus embriogenik bagi kedua-dua spesies dan benih berjaya untuk terus hidup selama 3 bulan. Semua anak pokok yang terhasil telah berjaya menyesuaikan diri dengan 91.07±0.96% kadar kemandirian untuk *C. ternatea* L. dan 71.58±1.27% kadar kemandirian untuk *O. viciifolia* Scop. Oleh kerana kepentingan ciri-ciri morfologi untuk pertimbangan taksonomi, analisa FESEM dan histologi mendedahkan kehadiran lubang cetek stomata dan trichome bukan kelenjar dengan hujung mata tajam, sama seperti pokok induk yang

menunjukkan tiada berlakunya variasi somaclonal. Spesis ini memberi respon positif terhadap bakteria (*Bacillus subtilis*, *Staphylococcus aureus* dan *Escherichia coli*) dan kulat (*Fusarium* sp., *Candida* albican dan *Trichoderma* sp.). Sebagai potensi penggunaan dalam teknologi cat, ia telah mendapati bahawa anthocyanin daripada kelopak biru *C. ternatea* L., berupaya menahan pH, UV, NaCl dan haba, Secara amnya, nilai keserapan anthocyanin daripada *C. ternatea* L. menurun apabila diuji dengan pH yang lebih tinggi, tempoh sinaran UV yang lebih lama, kepekatan NaCl yang lebih tinggi dan pendedahan haba yang lebih lama (pada 50±1ºC). Warna anthocyanin ini dipengaruhi oleh pH (merah pada pH 1, ungu pada pH 4.5, biru pada pH 5.5 dan cyan pada pH 6.5) dan menjadi lebih gelap dalam ketuhar (pada 50±1ºC) apabila tempohnya meningkat. Penambahan 1% asid tartaric kepada anthocyanin-PMMA telah menjadi penstabil terbaik untuk menambaik baik kestabilan dan ketahanan warna tersebut. Secara keseluruhannya, *C. ternatea* berupaya menyesuaikan diri di dalam kedua-dua iklim tropika dan sederhana tanpa mengira kekangan geografi dan musim untuk pengeluaran komersial disepanjang tahun.

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LIST OF ABBREVIATIONS

ANOVA	: Analysis of variance
$ADSO_4$	: Adenine hemisulfate
BAP	: Benzylaminopurine
$CaCl_2.2H_2O$	: Calcium chloride dehydrate
CMC	: Carboxymethycelulose
g	: gram
FESEM	: Field Emission Scanning Electron Microscope
HCl	: Hydrochloric acid
KIN	: Kinetin
L	: liter
cm	: centimeter
mg/L	: miligram per liter
MS	: Murashige and Skoog
NAA	: Naphtalene acetic acid
NaOH	: Sodium hydroxide
NaC6H7O6	: Sodium alginate
PGR	: Plant growth regulator
pH	: power of hydrogen
SE	: standard error
SPSS	: Statistical Packege for Social Science
Tween 20	: Polyoxyethylene sorbitan monolaurate
w/v	: weight per volume
2-ip	: 2-isopentenyl adenine
2,4-D	: 2,4-dichlorophenoxyacetic acid

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CHAPTER 1

INTRODUCTION

1.1 RESEARCH BACKGROUND

The history of plant tissue culture can be traced back as early as 1838 when Schleiden founded the 'Cell Theory'. According to this theory, every single cell is autonomic and thus, it is capable of developing into a complete individual (totipotency). Plant tissue culture refers to the growth and development of cells, tissues and organs which has been isolated from the mother plant and cultured *in vitro* on culture media under aseptic conditions (George, 1993). It provides efficient *in vitro* micropropagation methods for many economically important plant species (Gamborg et al., 195) and the most important prerequisite for successful genetic transformation (Slater, 2003). According to Thorpe (2013), plant tissue culture is a useful tool for the study of cell behavior, which includes morphogenesis, primary metabolism, secondary metabolism, cytology and pathology of cultured tissues. These studies can be applied for five broad areas, namely: (1) cell behaviour, (2) plant modification and improvement, (3) pathogen-free plants and germplasm storage, (4) clonal propagation, and (5) product formation. The micropropagation consists of two fundamental morphogenesis processes (organogenesis and somatic embryogenesis). Organogenesis is the formation of plant organs from a determined tissue into a new complete plant, whilst somatic embryogenesis is the production of embryos from somatic plant cells to obtain a complete plant. Among the advantages of this technique is good quality of planting materials with disease and virus free at a competitive price while conserving these plants in the natural habitat. Large scale plant production can also be programmed and preservation of plant species *in vitro* is also possible. *In vitro* propagation is the most efficient and cost effective method of propagating large number of planting materials.

1.2 PROBLEM STATEMENT

The plants produced by *in vitro* propagation are expected to be genetically uniform, vigorous and free from associations with microorganisms and useful for the culture of plants where contaminating organisms can dominate other types of production systems (Ailstock and Shafer, 2006). Therefore, tissue culture technique which is done under controlled conditions and temperatures is a suitable method to mass propagate *Clitoria ternatea* L. (tropical legume) and *Onobrychis viciifolia* Scop. (temperate legume) without depending on particular weather of regions or countries. In the present research, *C. ternatea* L. is a leguminous woody plant that known in difficulty to regenerate under *in vitro* conditions. Previously, the commonly explants used are leaf, cotyledonary node nodal, petiole and stem explants cultured on MS media for plant regeneration (Mukhtar, 2010; Pandeya et al., 2010; Ismail et al., 2012). Previously, *Onobrychis viciifolia* Scop. Was regenerated from stem, petiole, leaf, embryogenic axis and cotyledon node explants (Ozgen et al., 1998; (Sancak, 1999; Saglam, 2010. Due to the increasing demand for nutritive forage purposes (particularly in Malaysia), as well as prior to successful agri-biotechnological research on crops, an efficient *in vitro* regeneration system is urgently required (Abdellatef et al., 2008). To date, there was no report on *in vitro* regeneration of *C. ternatea* L. and *O. viciifolia* Scop. from root explant cultured on MS media supplemented with combinations of adenine hemisulfate ($ADSO_4$), naphtalene acetic acid (NAA), benzyaminopurine (BAP), as well as 2,4- dichlorophenoxyacetic acid (2,4-D) and kinetin (KIN).

1.3 RESEARCH OBJECTIVES

Malaysia (2° 30’ North latitude and 112° 30’ East longitude) is a tropical country with high temperature (24°C-32°) and high humidity throughout the year. However, some places in highland areas such as Cameron Highlands, Genting Highlands, Bukit Tinggi, Fraser Hill and Ranau have lower temperatures (17°C-25°C). Geographically, Cameron Highlands, Pahang, is located on the highland spine or Main

Range (Banjaran Titiwangsa) of Peninsular Malaysia, which the height ranged 1070-2031 m from sea level. The areas are mainly focused for cultivation of temperate vegetables (*Brassica oleracea* botrytis and *Citrus assamensis*) flowers (*Clitoria ternatea*, *Oxalis triangularis*, *Lawsonia inermis*, *Phlox paniculata*, *Celosia plumosa*, *Celosia cristata*, *Canna indica*, *Echinocereus cinerascens*, *Agapanthus praecox*, *Justicia betonica*), grasses (Australian blue couch, carpet and Guinea grass) and tea plantation, as well as for rare and elite species such as fern (*Cibotium barometz*)and other potential local and imported species, even some aquatic plant (*Nelumbo nucifera* and *Oryza sativa*). Generally, in Cameron Highlands, the mean annual temperature is about 18-25°C during day time, while at night, it can drop as low as 13°C. In Kundasang, Ranau, Sabah (near to Mount Kinabalu, 4905.2 m from sea level ('The highest mountain in South East Asia'), 'Mini New Zealand' with 'Desa Dairy Farm' for milk and meat, that feed with fresh grown imported forage crops such as Kikuyu, Narok setaria and white clover, also *Medicago sativa* or alfafa ('Queen of Forages'), *Trifolium* sp. (clover) and *Onobrychis* sp. (Sainfoin) and tea. Generally, lower temperature (mean 3-15°C) mimics to the autumn season in temperate countries, which help the plants grow healthier with high quality of leaves, flowers and fruits. The most important characteristics of Leguminosae are deep roots such as in *C. ternatea* L. and *O. viciifolia* Scop. which are beneficial for organic farming which can increase the fertility of surface soil by extracting nutrients from deeper layer of soil and depositing them on the surface in the form of organic matter. Besides fixing nitrogen (soil improver) and avoid soil erosion (as cover crop), ultimately as constituent of green technology with low labour cost.

The main objectives of this research were:

1. To investigate and compare seed germination of *C. ternatea* L. (tropical legume) and *O. viciifolia* Scop. (temperate legume) at different temperatures (18, 24 and 30±1°C), under *in vivo* and *in vitro* growth conditions.

2. To establish *in vitro* regeneration systems (for shoots, roots and callus formation) of *C. ternatea* L. and *O. viciifolia* Scop. using MS media supplemented with various hormones ($ADSO_4$, NAA, BAP, 2,4-D and KIN) from root, stem and flower bud explants.

3. To determine the optimum concentrations of sodium alginate ($NaC_6H_7O_6$) and soaking period in calcium chloride dehydrate ($CaCl_2.2H_2O$) on synthetic seeds production from embryogenic callus of *C. ternatea* L. and *O. viciifolia* Scop.

4. To study and compare the acclimatization and morphological characteristics of *C. ternatea* L. and *O. viciifolia* Scop. through Field Emission Scanning Electron Microscope (FESEM) and histology analysis, grown *in vivo* (mother plant) and *in vitro* (regenerants).

5. To investigate the antimicrobial activity from *in vivo* leaves, *in vitro* leaves and callus of ethanolic extracts of *C. ternatea* L. and *O. viciifolia* Scop. against bacteria (*Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli*) and fungi (*Fusarium* sp., *Candida albican* and *Trichoderma* sp.).

6. To evaluate the potential of anthocyanin and stability from vivid blue petals of *C. ternatea* L. as a natural colorant.

CHAPTER 2

LITERATURE REVIEW

2.1 GENERAL INTRODUCTION

2.1.1 Introduction to Tissue Culture Technique

2.1.1.1 *In Vitro* Regeneration

The *in vitro* propagation or plant tissue culture is an alternative method in producing new plants (Chu and Kurtz, 1990) with addition of Plant Growth Regulators (PGR) such as auxin and cytokinin. Generally, root formation occurred with addition of auxin (such as IAA, IBA, NAA and 2,4-D) at lower concentration, whereas higher concentration will promotes callus formation. Meanwhile, cyttokinins (such as BAP, KIN and zeatin) will promote the shoot formation (Pierik, 1987). Through this cloning technique, plants with genetically identical to mother plant (true-to-type) can be produced through multiplication of small pieces of tissues (Bhojwani and Radzlan, 1983). Pierik (1987) reported that plants can propagate through sexual (generatively) or asexual (vegetatively). The development of plant tissue culture is highly related to the chemical constituents which are the growth regulators (Skoog and Miller, 1957). Therefore, it is important to determine the balance medium that is suitable for the growth of tissues from a particular plant type.

2.1.1.2 Synthetic Seeds Production

Traditionally, germplasm has been maintained as seed, but the ability to regenerate whole plants from somatic and gametic cells has led to their use for storage (Bhojwani and Razdan, 1983). *In vitro* propagation is a viable alternative for a species which is difficult to regenerate by conventional methods; where populations have decreased due to over exploitation by destructive harvesting and can effectively be used to meet the growing demand for clonally uniform elite plants.

Therefore, the measures to develop micropropagation protocols for elite stocks are urgently needed. The development of encapsulated or synthetic seed technology brings up a new prospect in agriculture and floriculture industry.

Initially, synthetic seeds technology consist of propagules produced only from alginate encapsulated somatic embryos originating from rapidly multiplying vegetative cell cultures (Murashige, 1978). The synthetic seed is an artificial endosperm gel containing nutrients consist of sodium alginate salt that encapsulated around the propagules. This technology has expanded to include propagules from other totipotent tissue such as leaf, shoot, root and stem (Standardi and Piccioni, 1998) and suitable for plants that not producing seeds, hard to germinate or endangered. Aitken-cristie et al. (1995) reported that synthetic seeds can be sown under both conditions, *in vitro* (aseptic) and *ex vitro* (non-aseptic). The synthetic seeds technology beneficial in storage, transportation and viability with low cost of production (Gosh and Sen, 1994). Previously, this technology was successfully used for germination such as *Solanum melongena* (Huda et al., 2007), potato (Sarkar and Naik, 1998), *Brassica oleraceae* (Siong, 2012) and *Oryza sativa* (Taha, 2012). Synthetic seeds consist of artificially encapsulated somatic propagules (embryos, shoots or other *in vitro*-derived propagule) capable of conversion into plantlets *in vitro* or *ex vitro* conditions (Aitkenchristie et al., 1995). Encapsulated technology using *in vitro*-grown propagules offers an efficient means for mass clonal propagation and conservation of elite or endangered plant species (Mallón et al., 2007). Generally, there are three different synthetic seeds systems, namely; 1) The production of synthetic seeds through single encapsulation in beads or droplet hardening method consisting of MS salts plus 1% (w/v) sodium alginate ($NaC_6H_7O_6$) which was dropped into 100 mM calcium chloride dehydrate ($CaCl_2$) solution for 20 min for hardening (Redenbaugh et al., 1993). 2) The development of the technique using pharmaceutical capsules which filled with liquid

MS salts and the microshoots (Dupuis et al., 1994). 3) The double layer or hollow beads techniques based upon production of capsules with a gelled external layer surrounding a liquid internal layer capsule formation using carboxymethylcelulose (CMC) in $CaCl_2$ 100 mM solution for 20 min, which then immersed in a solution of 1% $NaC_6H_7O_6$ before drooped in a $CaCl_2$ solution and hardened for 20 min (Patel et al., 2000). The main advantages using synthetic seed techniques are associated with the elimination of the final micropropagation stages (rooting and acclimatization) allowing direct delivery as well as the development of cost-effective propagation systems (Singh et al., 2006). Synthetic seeds derived from microshoots or somatic embryos of superior hybrids can be produces from very small pieces of plant tissue, to serve as a supplement to the already existing propagules, to prevent extinction of endangered species, to increase productivity and to propagate plants with low seed viability and expensive imported seeds. Synthetic seeds can also be used for cryopreservation of elite genotypes to either export or import such as for *C.ternatea* L. and *O. viciifolia* Scop.

2.1.1.3 Acclimatization and Micromorphological Characteristics

Acclimatization is an adaptation process to the natural environment for various plant species which has undergone growth and development process *in vitro* (Preece and Sutter, 1991). In tissue culture system, most of the acclimatized *in vitro* plantlets showed almost similar characteristics as intact plants (Mohammed and Vidaver, 1990). In acclimatization stage, management of light intensity, substrate moisture and temperature at the leaf and root level are strongly recommended and can influence plant survival rate (Garcia-Gonzáles et al., 2010). According to Pospóšilová etal., (1999), at the beginning of acclimatization stage, a constant high relative humidity is recommended to facilitate the formation of active roots and to reduce water lost by transpiration, but as plants start to adapt it is recommended to decrease the humidity in order to facilitate a better adaptation to natural environment

condition. Generally, micromorphological aspects of the leaf surface are influenced by habitat and adaptation, as well as the exposure of sunlight. The importance of micromorphological features are for the taxonomic consideration or identification (Parveen et al., 2000) and micromorphological aspects of the leaf surface are influenced by habitat and exposure of sunlight for adaptation using electron and histology analysis. According to Kathiresan et al. (2011), the micromorphological parameters of different plant parts have been used as aids in the taxonomical recognition of species. The foliar epidermis (including leaf), is one of the most noteworthy taxonomic characters from a biosystematics point of view. Size, distribution, and frequency of stomata have been found to be specific to taxa and are used as significant parameters in taxonomy as well as in elucidating phylogeny (Ahmed, 1979; Rajagopal, 1979; Idu et. al., 2000; Barkatullah et. al., 2014).

2.1.1.4 Antimicrobial Properties

Due to antimicrobial-resistant organisms have been the major problem in medical treatment, searching for new antimicrobial compounds are still is interested. During the past 15 years, a large number of antimicrobial proteins (AMPs) have been identified in different plants (Broekaert et al., 1997). AMPs constitute a heterogenous class of low molecular mass proteins, which directly interfere with the growth, multiplication and spread of microbial organisms (Lehrer and Ganz, 1999). Different proteins with antibacterial and antifungal activity have been isolated from seeds, tubers, and rhizomes in floral and vegetative tissues (Terras et al., 1995; Kheeree et al., 2011; and Charungchitrak et al., 2011). Plants can produce antibacterial and antifungal compounds from leaf extraction for protection from biotic attack that could be esential for infection resistance (Wotjaszek, 1997). There are several classes of proteins having antimicrobial properties which include thionins, lipid transfer proteins, plant defensins, chitinases, glucanases, 2S albumins, ribosome inactivating proteins and lectin (Ye et al., 2002;

Zhang and Halaweish, 2003; Sharon and Lis, 2001). Legumes and monocots are major sources of plant lectins that have been widely studied (Wood et al., 1999), whereas the seed lectins are particularly seen in cotyledons where they appear during the later stages of maturation of the seeds (Howard et al., 1972). During the early seedling growth, Weber and Neumann (1980) noticed the decrease in lectin concentration as the cotyledons are resorbed. In Leguminosae, seed lectins are confined to cotyledons for up to 5% of the total seed proteins. The non-seed lectins are found in all kinds of vegetative tissues such as leaves, stem, bark, bulb, tubers, corns, rhizomes, roots, fruits, flowers, ovaries, phloem sap and even in nectar (Peumans and Van Damme, 1995).

Despite the abundance of antibiotics used in chemotherapy, there is a drastic increase of resistant bacteria and fungi. Resistance to antibiotics occurs typically as a result of drug inactivation or modification, target alteration, or reduced accumulation associated with decreased permeability or increased efflux (Poole, 2002). Plants are also rich in a wide variety of secondary metabolites polyphenols, such as tannins, terpenoids, alkaloids, and flavonoids, which have been demonstrated to have *in vitro* antimicrobial properties (Gonzalez-Lamothe et al.,2009). The variety of compounds produced in *in vivo* and *in vitro* plants can show different bioactivity potentials and this is similar with the bioactivities, it will differ between *in vitro* and *in vivo* grown plants. Antimicrobial activities differ in *in vivo* and *in vitro*, probably due to the inherent characteristics of the fully grown plants and the maturity of its chemically active constituents.

In 1999, 25-50% of pharmaceutical products are derived from natural sources, however, it was reported that none are used as antimicrobial (Cowan, 1999). Moreover, there is considerable interest of manipulating pigments content and compositions in plants to improve the agronomic and nutritional value for human and animal consumption (Cunningham and Gantt, 1998). Therefore, improving

nutritional quality of food crops and its ingredients for human consumption is one of the urgent health issues and high priority areas of research worldwide (Hui and Khacatourians, 1995; DellaPenna, 1999).

2.1.1.5 Anthocyanins Extraction

Plant tissue culture system represent a potential renewable source of valuable natural extracts for medicinal, flavours, essences and colourants. Pigments from plants contain components of therapeutic that have been used as remedies for human diseases for centuries. According to Schoefs (2004), natural pigment can be classed into four families involving tetrapyrroles (e.g. chlorophyll), carotenoids (e.g. beta-carotene), polyphenolic compounds (e.g. anthocyanins), and alkaloids (e.g. betalains). Natural pigment extracts from medicinal plants are not associated with side effects and have enormous therapeutic potential to heal many infectious diseases and can be developed as better new drugs against microbial infection (Benkeblia, 2004).

Anthocyanins are water soluble pigments which responsible responsible for the particularly red, orange, blue and purple. Anthocyanin content has a critical role in the colour quality of coloured leaves (Onslow, 1916), flowers (Rajendran, 2010) and fruits (Lohachoompol et al., 2004). According to Janna et al. (2006), the highest anthocyanin concentration is reported to be present in the closed full-formed petals. Moreover, anthocyanin beneficial in reduction of coronary heart disease (Bridle and Timberlake, 1996; Bell and Gochenaur, 2006; Hidalgo et al., 2011), anti-inflammatory (Xia et al., 2007), anti-microbial (Puuponen-Pimia et al., 2005), antioxidant (Takamura and Yamagami, 1994; Wang et al., 1997; Fukumoto and Mazza, 2000; Ramawat and Me'rillon, 2013), anticancer (Karaivanova et al., 1990; Kamei et al., 1995; Liu et al., 2005), improved visual acuity (Timberlake and Henry, 1988; Canter and Ernst, 2004; Fursova et al., 2005), stimulate insulin for diabetic (Jayaprakasam et al., 2005) and Alzheimer's disease (Joseph et al., 2003).

In Leguminosae, there are more than 60 different anthocyanins have been identified including 13 novel anthocyanins from *Clitoria ternatea* (Terahara et al., 1996; Kazuma et al., 2003). Marko et al. (2004) reported the most anthocyanins commonly found including pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin (Figure 1.1). Anthocyanins have been used as natural colourant for food and beverages; however, many anthocyanins are unstable in neutral solutions (Dougall and Baker, 2008). According to Parisa et al. (2007), the stability of anthocyanin pigment is depends on various factors including structure and concentration of anthocyanin, pH, temperature and presence of complexing agent. Therefore, anthocyanins have a useful potential as natural colorants (Markakis, 1982) and beneficial health effects (Torskangerpoll and Andersen, 2005).

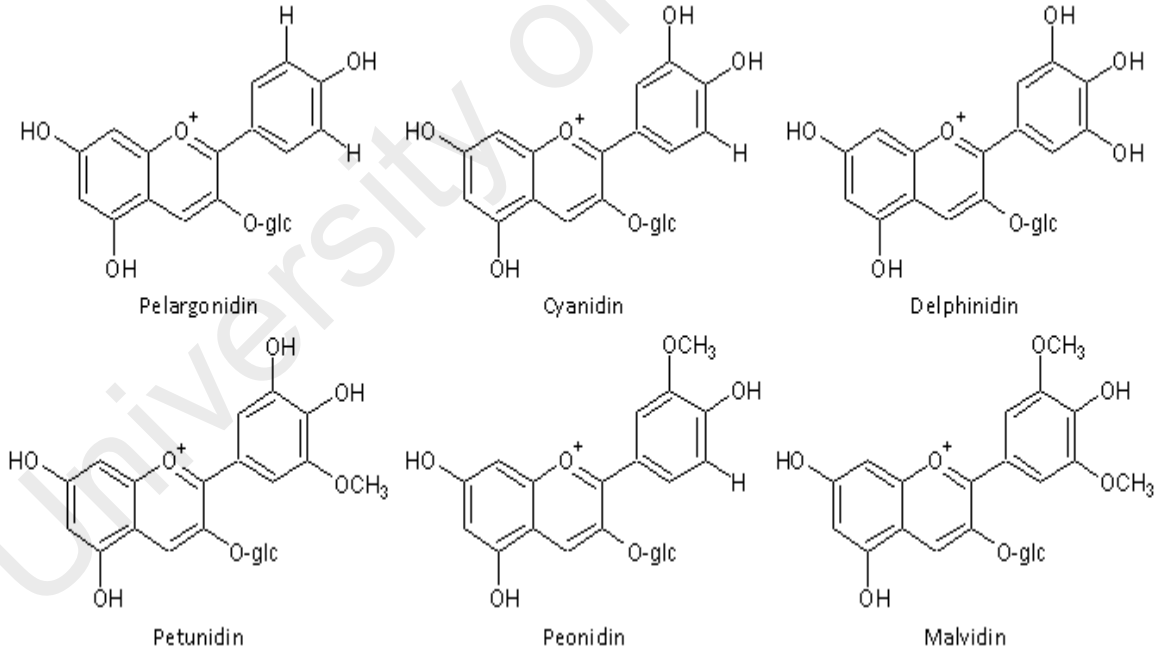


Figure 2.1 : Chemical structures of anthocyanins.

2.1.2 Introduction of Leguminosae (Fabaceae)

The Leguminosae or Fabaceae (pea family), is the third largest family of angiosperms which consist of approximately 750 genera and 19 000 species of trees, climbers, herbs and shrubs, which are divided into three subfamilies

(Mimosoideae, Caesalpinoideae and Papilionoideae), which vary to shrubs, trees, vines and a few aquatic plants. The characteristics particularly of the flower (including size, symmetry, aestivation of petals, sepals structure, stamen number and heteromorphy, pollen structure, as well as the presence of a pleurogram, embryo radicle shape, leaf complexity and presence of root nodules (Lewis et al., 2005).

Most of the legumes are valued as sources of protein and constitute as one of the main non-animal proteins in the daily diet. Nutritionally, legume seeds such as *C. ternatea* L. (tropical legume) and *O. viciifolia* Scop. (temperate legume), are two to three times richer in protein as compared to cereal grains. Therefore, both species are commonly supplemented in grass-legume mixtures as the main protein source to increase the livestock meat and dairy quality.

Legumes, including *C. ternatea* L. and *O.s viciifolia* Scop. are very important in agriculture, industry and environmental due to the root systems that are able to fix nitrogen . According to De Faria et al. (1989), 88% of the species form nodules with *rhizobia* to fix nitrogen naturally. In Leguminosae, the bacteria live in small growths on the roots called nodules. This symbiotic system between bacteria and nodules will convert the nitrogen gas (N_2) to ammonia (NH_3). Other plants, as well as soil, benefited from N-fixing bacteria when the bacteria die and release nitrogen to the environment, or when the bacteria live in close association with the plant.

2.1.2.1 Characteristics of Leguminosae

Leguminosae (Fabaceae) was divided into three different families based on the leaf and flower structures. Most legume leaves are pinnately or palmately compound, but some are simple. The flower petals are irregular with five petals forming a distinctive banner, wings and keel (with bilateral symmetry) and had been described as either butterfly or boat shape. The banner (first outer petal) is a single petal with two lobes, followed by other two petals form wings and the remaining two petals make

up the keel. The fruit is a pod, or legume, with one row of seeds or peas that contain two prominent food-storing cotyledons.

2.1.2.2 Advantages of Leguminosae

Leguminosae was recognized as multi-purpose plants due to the nutritional and contribution to agricultural, industrial and environmental as listed below:

1. Protein-rich Nutritional

Legumes are important food worldwide due to the higher protein content compared with other food plant and are close to animal meat in quality. Protein quality for *O. viciifolia* is around 68 (19-21% crude protein) as compared to 71 (20-22%) for alfafa ('Queen of Forages') out of a possible score of 100 for an 'ideal protein' (Kaldy et al., 1979).

2. Biological Nitrogen Fixation

Nitrogen (N) is the primary nutrient limiting plant production in most natural ecosystems (Vitousek et al., 1997). Approximately, 80% of Earth's atmosphere contain nitrogen gas (N_2) which is cannot be used directly by living organisms. All organisms use the ammonia (NH_3) form of nitrogen to manufacture amino acids, proteins, nucleic acid, etc. This biological nitrogen fixation is mediated in nature only by N-fixing *rhizobia* bacteria (*Rhizobiaceae*). Arianoutsou and Thanos (1996) reported that legumes, through their symbiotic abilities, play an important role in colonizing disturbed ecosystem to fix nitrogen from atmosphere, including those that are fire-prone. Formation of symbiotically effective root nodules involves signaling between host and microsymbiont. Flavonoids and isoflavonoid released from the root of the legume host induce transcription of nodulation compatible rhizobia, leading to the formation of lipochitooligosaccharide molecules that, in turn, signal the host plant to begin nodule formation (Long, 1996). Nitrogen (N) from legume fixation is essentially "free" N for use by the host plant or by associated or subsequent crops.

3. Leguminous Cover Plants

The important of cover plants are to prevent soil erosion, as well as leaching which causes losses of nutrients. These plants also reduce competition from noxious weeds. Later will enrich the soil with organic matter, which indirectly improve and fertilize the soil structure for better aeration, infiltration and retention of moisture, as well as reducing soil temperature. According to Hartley (1977), when selecting ground cover plants, preference is always given to legumes because they fix nitrogen and make it available to the main crop. When the legumes died, it will fertilized the soil (green manure).

4. Agricultural Advantages

Among the advantages of leguminous cover plants are as the high level of phenolic compounds, non-palatability to cattle and has the potential to increase animal feed availability and soil fertility. The integration in cropping system (such as palm oil-*C. ternatea* L. or *O. viciifolia* Scop.-cattle). Cultivated forages are of better-feed quality for ruminants compared to weed fallows (Carr et al., 2005).

5. Industrial Advantages

Legumes are most valuable in agricultural and industrial purposes as shown in Table 2.1. In addition to food source, specific legumes are valued as timber (*Leucaena leucocephala*), forage (*Onobrychis viciifolia*), spices and ornamental (*Clitoria ternatea*), as well as potent to be introduced as industrial products (medicines, insecticides, resins and dyes). Legumes have been used industrially such as in biodegradable plastics (Paetau et al., 1984) and also preparation of oils, gums and inks (Morris, 1997). According to Garcia et al. (1998), liquid form of some legumes can produce milk, yogurt and infant formula, as well as pop beans (Popenoe et al., 1989), licorice (Kindscher, 1992) and soybean candy (Genta et al., 2002).

Table 2.1 : The multi-purpose importance of Leguminosae as illustrated by Levetin-McMohan (2008).

Legumes	Purpose
Leucaena leucocephala (Leadtree)	Timber
Dalbergia spp. (Rosewood)	
Derris elliptica (Tuba-root)	Insecticide
Abrus precatorius (Rosa pea)	Accessories
Trigonella foenum-graecum (Fenugreek)	Spice
Glycine max (Soybean)	Milk, tofu, infant formula, Lecithin (additive)
Arachis hypogea (Peanut)	Oil
Cassia fistula (Senna pods)	Laxative
Ceratonia siliqua (Carob)	Chocolate subsitute
Copaifera officinalis (Copaifera)	Resin for paints, lacquers
Indigofera tinctoria (Indigo)	Dye
Clitoria ternatea (Asian Pigeonwings)	
Glycyrrhiza glabra (Licorice)	Medicinal extract
Prosopis glandulosa (Mesquite)	Charcoal
Tamarindus indica (Tamarind)	Seasoning
Medicago sativa (Alfafa)	Forage
Onobrychis viciifolia (Sainfoin)	

6. Medicinal Purposes

Leguminosae, world widely known as one of the major sources of medicinal herbsin China, especially on astragalus and licorice. These species valued for lecithin (oil), vitamin, protein and flavonoid that contributed to humankind health. *C. ternatea* L. has been recognized as one of the multi-purpose medicinal legume plants.

2.2 COMPARISON BETWEEN *Clitoria ternatea* L. (TROPICAL LEGUME) AND *Onobrychis viciifolia* Scop. (TEMPERATE LEGUME)

2.2.1 Classification and Nomenclature of *Clitoria ternatea* L. and *Onobrychis viciifolia* Scop.

C. ternatea L. or its common names including Bunga Talang (Malaysia), Tembang Telang (Indonesia), Anchan (Thailand), Cunhã (Brazil), Shankupushpam (India), Asian Pigeon Wings (England) and Butterfly Pea (Australia), belongs to the family Leguminosae (Fabaceae). *O. viciifolia* Scop. (Sainfoin) or commonly known as ‘Holy clover’ or ‘King of Forages’, is a perennial forage legume with a

deep taproot that allows the plant to be very resistant or tolerant to drought, requires an adequate supply of potassium to sustain forage yields and grow well on calcareous soils with low phosphorus, at pH 6.0. Morphologically and taxonomically (Table 2.2), Leguminosae is characterized by leaves and flowers structures. The uniqueness feature of the family is the fruit, the legume. Ranging in habit from large trees to annual herbs, the family is cosmopolitan in distribution and well represented throughtout temperate and tropical regions of the world (Rundel, 1989).

Table 2.2 : Classification and distribution of *Clitoria ternatea* L. and *Onobrychis viciifolia* Scop.

Classification	*Clitoria ternatea* L. (tropical legume)	*Onobrychis viciifolia* Scop. (temperate legume)
Kingdom	Plantae (plants)	
Subkingdom	Tracheobionta (vascular plants)	
Superdivision	Spermatophyta (seed plants)	
Division	Magnoliophyta (flowering plants)	
Class	Magnoliopsida (dicotyledons)	
Subclass	Rosidae	
Order	Fabales	
Family	Fabaceae / Leguminosae (Pea family)	
Genus	*Clitoria* L. (Asian Pigeonwings)	*Onobrychis*Mill. (Sainfoin)
Origin	Ternate Island in Molluca Archipelago, Maluku, Indonesia (tropical region)	Europe and South Asia (temperate regions)
Distribution	Australia, Brazil, South and Central America, East and West Indies, China and Europe	France, Italy, Turkey

2.2.2 Economical and Nutritional Values of *Clitoria ternatea* L. and *Onobrychis viciifolia* Scop.

2.2.2.1 Economical and Environmental Values

The economical and environmental value of legumes including *C. ternatea* L. and *O. viciifolia* Scop. (Table 2.3) for semi arid to arid habitats is related to a nitrogen-demanding metabolism, which is thought to be an adaptation to climatically variable or unpredictable habitats whereby leaves can be produced economically and opportunistically (McKey, 1994). Legumes play an important

role in the terrestrial nitrogen cycle regardless of whether they form root nodules (Sprent, 2001).

Table 2.3 : Economical and environmental comparison values of *Clitoria ternatea* L. and*Onobrychis viciifolia* Scop.

Plant characteristics	***Clitoria ternatea* L. (tropical legume)**	***Onobrychis viciifolia* Scop. (temperate legume)**
Adaptation	Adapted to heavy clay and medium to high pH (4.5-8.7). Drought tolerance and low tolerance to salinity.	Adapted to high calcium (calcareous) and pH (6.6 -8.0) soils. Fairly drought tolerant and low tolerance to salinity.
Nutrition	High protein and palatability, up to 80% digestibility.	High protein, high palatability and its non-bloating (enrich in tannin) forage.
Cultivation	Required to replanting every 5-8 years. It has persisted for 14 years.	For good establishment and growth, sainfoin seeds must be scarify and inoculated with a special rhizobium just before planting. Required to replanting every 2-3 years. It has persisted for 5 years.
Agricultural advantages	1. Protein-rich in hay 23% and seeds (38-43%) legume as comparable to meats, can be eaten raw or cook. 2. An edible medicinal plant in India (flower, leaves, fruit, root). 3. Flower used as natural colorant for food and textiles in Asia (Malaysia, Thailand, China). 4. Pea-like pod as food source in Philippines. 5. As"tropical alfafa" that palatable forage crop in Australia, Brazil, Delta Nile, Sudan and Indonesia.	1. Protein bank 2. Popular as high palatable anti-bloating to ruminants ("holy clover"), due to high tannin contents (4-10%). 3. Honey production
Economic advantages	1. Mass consumption of leaves for animal's feed especially cattle, sheep, (due to high protein content). 2. Honey bee production 3. Low cost of cultivation (easy to maintain and persistent up to 12-15 years)	
Environmental contribution	1.Symbiotic nitrogen fixation from atmosphere. 2.Soil fertility. 3.Cover crop to avoid soil erosion. 4.Green manure technology.	

2.2.2.2 Nutritional Values

Legumes include a large number of domesticated species including *C. ternatea* L. and *O. viciifolia* Scop. harvested as crops for human and animal nutritional (Figure 2.2) and consumption for oils, fiber, fuel, fertilizers, timber, medicinals, chemicals and horticultural varieties (Lewis et al., 2005).

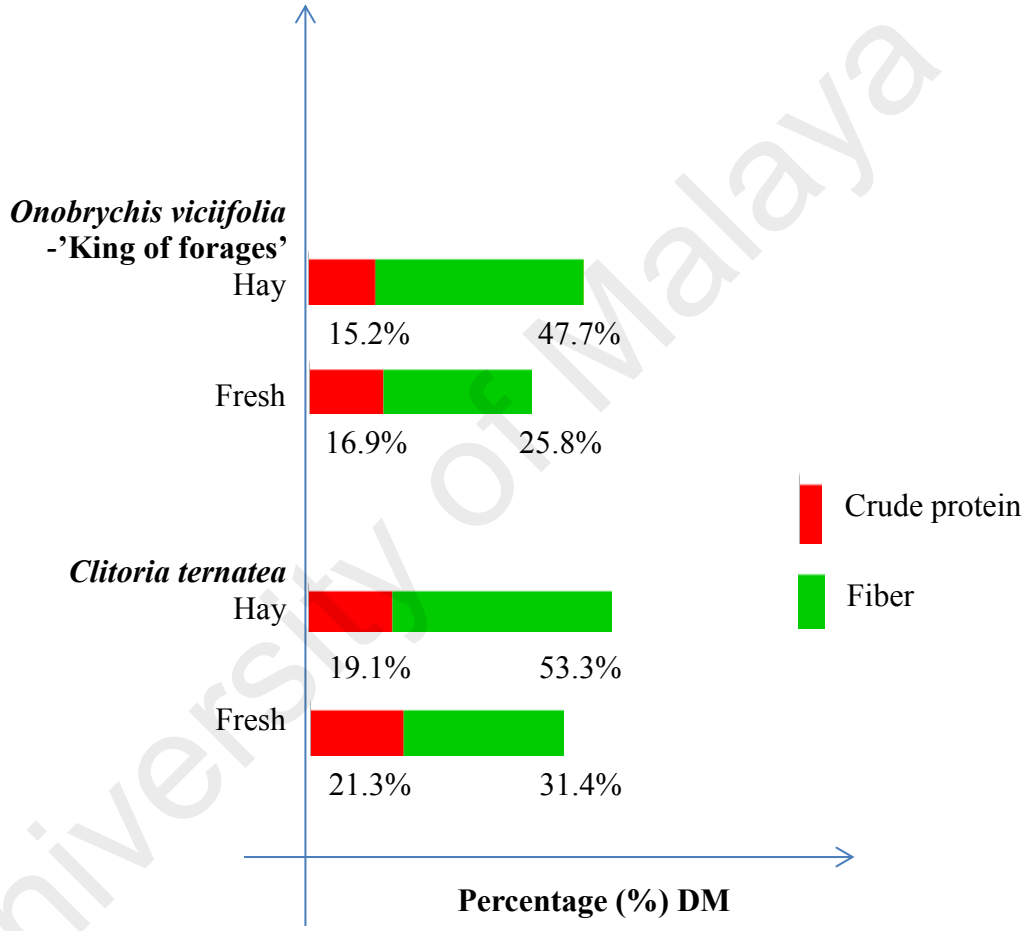


Figure 2.2 : Comparison of protein and fiber contents in forage crops (*Onobrychis viciifolia* and *Clitoria ternatea* (http://www.feedipedia.org)

2.3 INTRODUCTION OF *Clitoria ternatea* L. (TROPICAL LEGUME)

2.3.1 Description of *Clitoria ternatea* L.

C. ternatea L. (Figure 2.3) originated from Ternate Island in Molluca Archipelago, Maluku, Indonesia (tropical region), but is now naturalized in all the semi- arid and sub-humid tropics of Asia, Africa, Brazil and Australia (Staples,

1992). In Southeast Asia, *C. ternatea* L. is widely planted as an ornamental on fencerows and garden hedges (woody climber), as well as a natural food and textiles colorant (blue dye). *C. ternatea* L. is a perennial climber with twining fine stems (50-300 cm). The leaves are pinnate with 5-7 elliptic to lanceolate leaflets (3-5 cm) and shortly pubescent underneath. Flowers resemble a conch shell and solitary in vivid blue colour with very short pedicellate (4-5 cm). Pods are flat (6-12 cm), linear, beaked, slightly pubescent with up to 10 seeds. The mature seeds are olive shape in black colour (0.5-0.7 cm).

Figure 2.3 : *Clitoria ternatea* L. cultivation with vivid blue flowers in West Timor (Indonesia).
(Source: http://www.tropicalforages.info/key/Forages/Media).

2.3.2 Economic Importance of *Clitoria ternatea* L.

2.3.2.1 Edible Plant as Food Source

Blue petals of *C. ternatea* L. are popularly used as natural food dye to make a famous and nutritious local dish known as 'Blue Nasi Kerabu' (Malaysia) and 'Blue Tea' (Thailand). While, in Philippines, the young pods are edible and used as vegetable. According to Terahara et al. (1996), petals of *C. ternatea* L. contain good source of anthocyanin. In Australia, *C. ternatea* L. is particularly valuable as protein-rich forage in heavy and shallow soils where *Leucaena leucocephala* cannot grow (Cook et al., 2005). Ratnawaty et al. (2013) reported that *Stylosanthes*

seabrana, *Clitoria ternatae* Millgara, *Clitoria ternatea* Q5455 and *Centrosema pascuorum* Bundey produced the highest digestible protein compared to *Centrosema pascuorum* Molle, *Macroptilium bracteatum* Juanita, *Macroptilium bracteatum* Cadaarga and Dolichos lablab in West Timor, Indonesia (Figure 2.3). In Brazil, *C. ternatea* (Cunhã), has a good potential to be cultivated under irrigation because it yields good quality fodder, especially if this legume is offered fresh and with a high content of leaves (Abreu et al., 2014).

2.3.2.2 Nutritional and Pharmaceutical Values

C. ternatea L. (Figure 2 .4) also contains flavonoids such as quarcetin, kaemferol, robinin and Clitorin. It also contains starch, tannin, resin and anthocyanins (Kapoor, 2001) and several glycosides including malvidin-3-β-glycoside, deiphinidin-3-β- glycoside (Srivastava and Pandey, 1977). According to Terahara et al. (1996) and Kazuma et al., (2003), thirteen novel anthocyanins from *C. ternatea* were identified.

In India, *C. ternatea* L. is widely used in 'Aparajita' (traditional medicine) to promote memory and intelligence, in addition it is helpful for healing eye infection, skin diseases, urinary problems, ulcers and has antidotal properties (Gomez and Kalamani, 2003). According to Uma et al. (2009), phytochemicals found from roots of *C. ternatea* L. were ternatins, alkaloids, flavonoids, saponins, tannins, carbohydrates, proteins, resin, starch, taraxerol and taraxerone.

Due to the increasing demand for medicinal (Table 2 .4) and nutritive forage purposes of *Clitoria ternatea* L. (Table 3), the United State Development Agency (USDA) intends to conserve *C. ternatea* L. along with 16 other leguminous species with potentially useful phytochemicals (Morris, 1996).

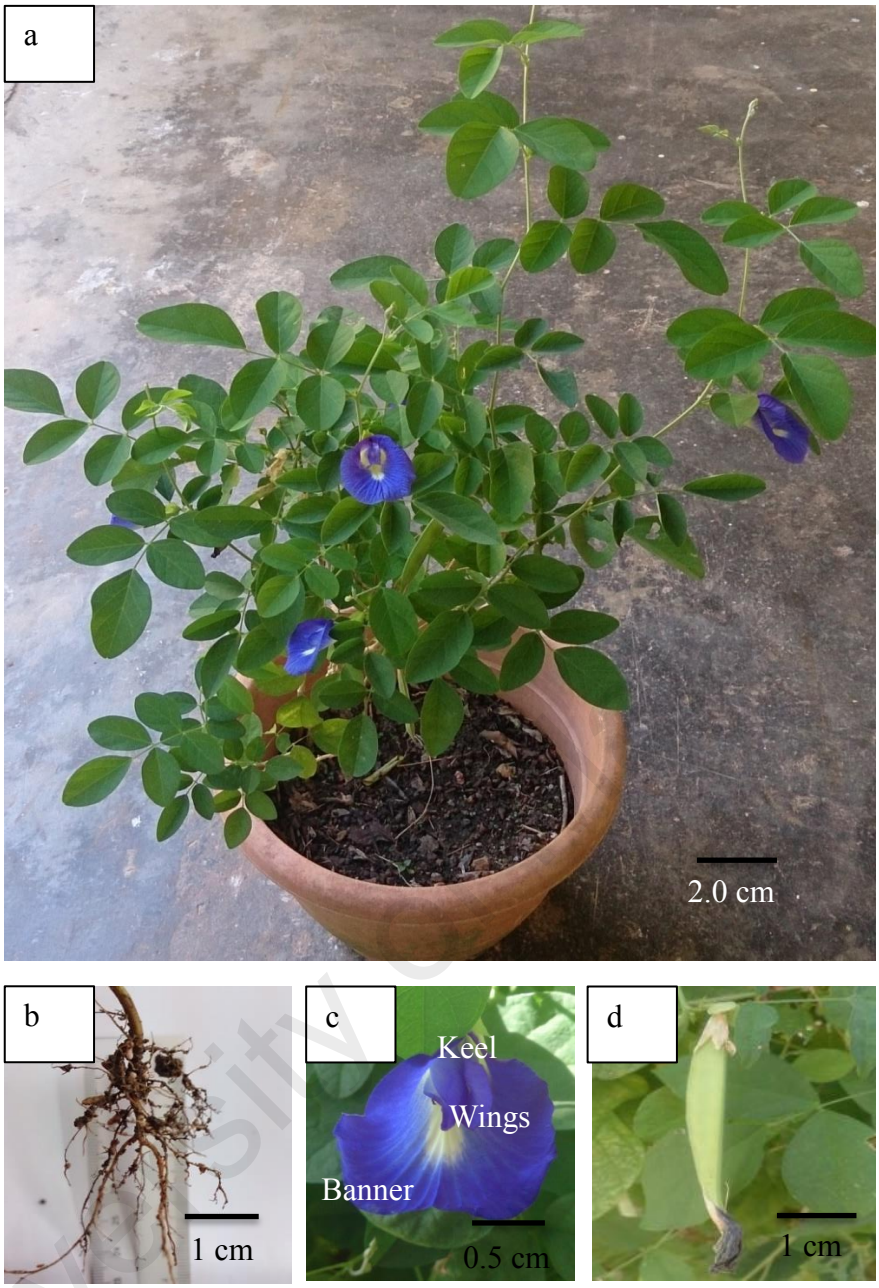


Figure 2.4 : Morphology of *Clitoria ternatea* L. a) Intact plant. b) Nodules attached to secondary roots. c) Butterfly-shape flower. d) Pea-like pod.

Table 2.4 : Phytochemicals reported in *Clitoria ternatea* L.

Part	Usage	Phytochemicals	Reference
Root	useful in asthma, burning sensation, ascites, inflammation, leukoderma, leprosy, hemicranias, amentia, pulmonary tuberculosis, ophthalmic laxative/ tonic.	Ternatins, alkaloids, flavonoids, saponins, tannins,carbohydrates, taraxerol and taraxerone.	Uma et al. (2009)
Leaf	reduced diabetes mellitus.	Kaempferol glycosides (I,II.III and IV/Clitorin	Mukherjee et al. (2008)
Seed	increase memory, reduce cholesterol and diabetes mellitus.	-delphinidin-3,3,5-triglucoside, -phenol glycoside, 3,5,7,4-tetrahydroxy-flavone rhamoglycoside, ethy D-galactopyranoside, p-hydroxycinnamic acid polypeptide, -g-sitosterol, β-sitosterol,hexacosanol , anthocyanin glucoside.	Sinha (1960)
Flower	to treat insect bites, skin diseases, scorpion sting and reduced diabetes mellitus	-delphinidin glycosides, anthocyanins, ternatins (C1,C2, C3, C4, C5, and D3), preternatins (A3 and C4) -malonylated flavonol glycosides -ternatins (A3, B3, B4, B2 and D2)	Terahara (1996)

2.4 INTRODUCTION OF *Onobrychis viciifolia* Scop.

2.4.1 Plant Description of *Onobrychis viciifolia* Scop.

O. viciifolia Scop. (Figure 2.5), or well known as 'Holy clover' or 'King of Forages' is a popular anti-bloating forage crop which are cultivated in most countries in parts of Europe and West Asia for hundreds of years (Ditterline and Cooper, 1975) and has been cultivated extensively in Eastern Europe (Sancak et al., 1999). Anonymous (2001) reported that over than 94000 ha of sainfoin is grown in Turkey, where over 800 ha in Erzurum, Turkey.

Figure 2.5 : *Onobrychis viciifolia* Scop. with pink coloured flower in Southern Europe. (Source:http://www.shutterstock.com).

2.4.2 Economic Importance of *Onobrychis viciifolia* Scop.

2.4.2.1 Forage Crop

O. viciifolia Scop. (Figure 2.6) is one of the most important forage crops which is appreciated in livestock agriculture due to its high palatability and nutritional value properties (Delgado et al., 2008). *O. viciifolia* Scop. contains condensed tannin in the herbage, which is known to be non-bloating legume compared to alfafa that is no tannin contain in the herbage, eventhough tannin present in the seed coat (Lees, 1992). The condensed tannin content of *O. viciifolia* Scop. appears to restrict protein breakdown not only in the rumen but also in the clamp as well (Wilkins and Jones, 2000). Sheep have improved utilisation of protein from sainfoin hay over lucerne hay (Thomson et al., 1971). *C. ternatea* L., similar to *O. viciifolia* Scop. is often grown in conjunction with forage grasses to reduce bloating grazing animals, as well as to improve soil fertility due to its nitrogen fixing ability.

In European countries, *O. viciifolia* Scop. is a multipurpose crop in production of bale and haw as forage crop, besides honey collection from the pink flowers (Figure 2.7). Therefore, this activities can be applied and commercialized in some regions in Malaysia (such as in Cameron Highlands and Kundasang), due to the low temperature (17°C-25°C).

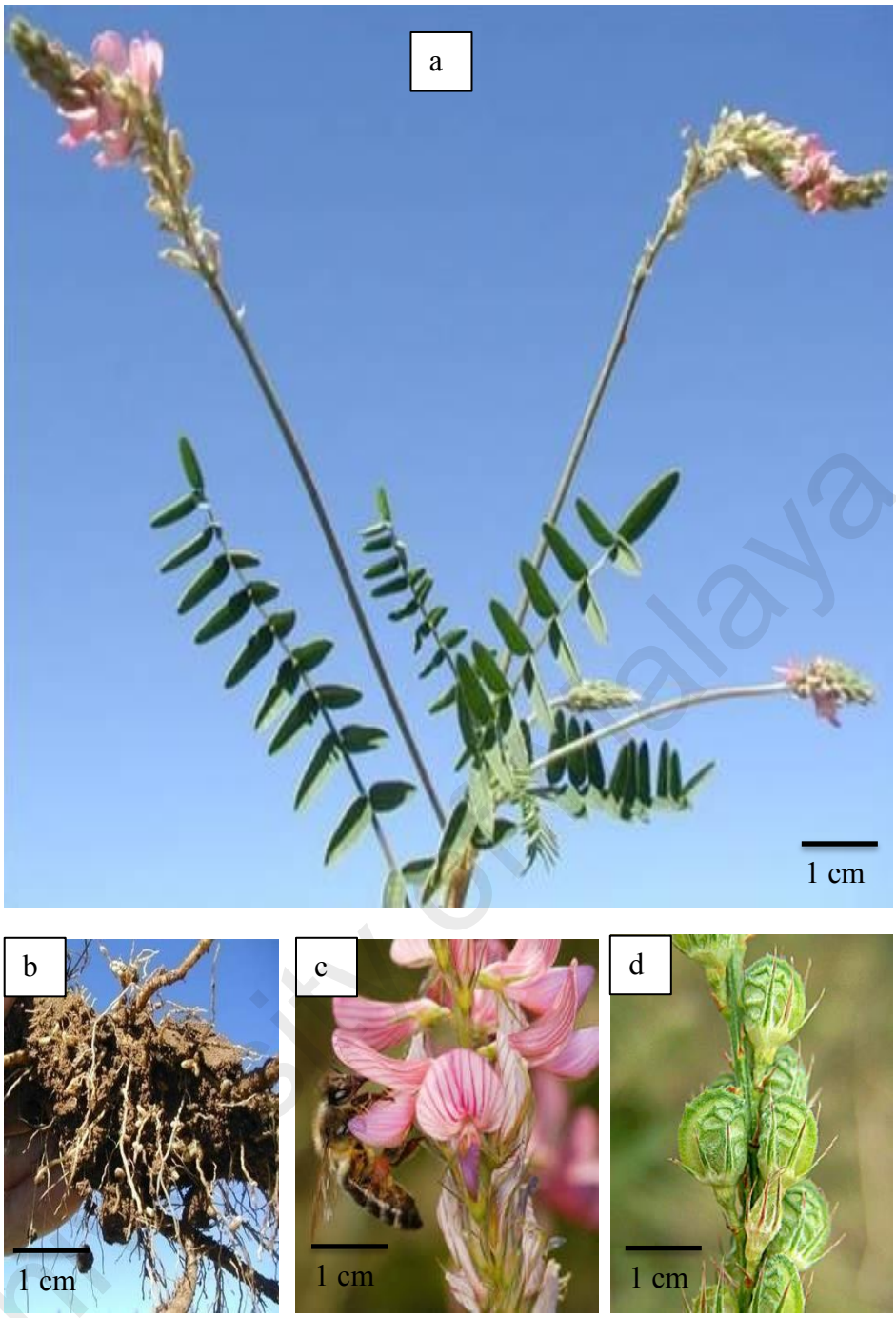


Figure 2.6 : Morphology of *Onobrychis viciifolia* Scop. a) Intact plant with pinnate leaves and hollow stem. b) Nodules attached to secondary roots. c) Pink cluster flower petals. d) Large seed. (Source:http://www.uwyo.edu/plantsciences/uwplant/forages).

Figure 2.7 : Established products by *Onobrychis viciifolia* Scop. in temperate regions that potentially to be introduced and adapted in Malaysia. a) Bale. b) Hay and straw. c) *Onobrychis viciifolia* Scop. or Sainfoin honey. d) Kundasang, Sabah. e) Cameron Highlands, Pahang. (Source:www.google.com)

CHAPTER 3

IN VITRO REGENERATION OF *Clitoria ternatea* L. (TROPICAL LEGUME) AND *Onobrychis viciifolia* Scop. (TEMPERATE LEGUME)

3.1 EXPERIMENTAL AIMS

Clitoria ternatea L. (tropical forage legume) and *Onobrychis viciifolia* Scop. (temperate forage legume), are perennial plants with deep taproots that allow the plants to be very resistant or tolerant to drought and salinity. Both species are important in livestock agriculture due to the nutritional and palatable properties, but naturally grow in different regions based on the temperatures, climates, soils and origin. Originally, these plants are selected as cover crops, but now they are popular for short and medium-term pastures, green manure and protein bank. *C. ternatea* L. is adaptable in both extended rainfall regions and prolonged periods of drought and actively in livestock and dairy industry especially in Australia, Indonesia and Brazil, *C. ternatea* is particularly valuable as highly digestable protein-rich forage and offered fresh and massive of leaves (high quality fodder), in heavy and shallow soils (Cook et al., 2005; Ratnawaty et al., 2013; Abreu et al., 2014). *O. viciifolia* Scop. or well known as 'Holy clover' or 'King of forages' contains condensed tannin in the herbage, which is known to be non-bloating legumes compared to the 'Queen of forages' (*Medicago sativa* or Alfalfa) that is no tannin content in the herbage, eventhough tannin present in the seed coat. The condensed tannin content of *O. viciifolia* Scop. appears to restrict protein breakdown not only in the rumen but also in the clamp as well (Wilkins and Jones, 2000). Sheep have improved utilisation of protein from *O. viciifolia* Scop. from hay over lucerne hay (Thomson et al., 1971). This legume forage crop is often grown in conjunction with forage grasses to reduce is bloating in grazing animals, as well as to improve soil

fertility due to its nitrogen fixing ability and surviving at calcareous soils with low phosphorus (pH 6).

Due to the increasing demand for nutritive forage purposes (particularly in Malaysia), as well as prior to successful agri-biotechnological research on crops, an efficient *in vitro* regeneration system is urgently required (Abdellatef et al., 2008). An i*n vitro* propagation is a viable alternative for a species which is difficult to regenerate (especially by temperatures barrier) through conventional methods; where populations have decreased due to over exploitation by destructive harvesting and can effectively be used to meet the growing demand for clonally uniform elite plants. Therefore, Malaysia as a tropical country with high (27-30ºC) and low (18-24ºC) temperatures such as in Cameron Highlands and Ranau, have potential to produce higher quality of *C. ternatea* L. and *O. viciifolia* Scop.

The main objectives of this specific chapter were:

1. To investigate seeds germination responses of *C. ternatea* L. and *O. viciifolia* Scop. at different temperatures (18ºC, 24ºC and 30ºC), under *in vivo* and *in vitro* growth conditions.
2. To study the effects of adenine hemisulfate ($ADSO_4$) on *C. ternatea* L. and *O. viciifolia* Scop. white callus in seed germination, root and stem explants (after 2-4 weeks).
3. To investigate *in vitro* regeneration system from root, stem and flower bud explants of *C. ternatea* L. and *O. viciifolia* Scop. using adenine hemisulfate ($ADSO_4$), naphthalene acetic acid (NAA), benzylaminopurine (BAP), 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (KIN).

3.2 MATERIALS AND METHODS

3.2.1 Plant Materials and Sterilization Methods

C. ternatea L. plants and seeds were obtained from nursery in Sungai Buloh, Selangor, Malaysia, while, seeds of *O. viciifolia* Scop. were imported from reliable websites online. The matured black seeds of *Clitoria ternatea* L. and *Onobrychis viciifolia* Scop. were in oval-shaped and initially washed with tap water and teepol. The seeds sterilization and disinfectant were based on standard protocols by Taha (1998), with minor modifications. The seeds were sterilized with 99% (v/v) chlorox (commercial bleach) solution for 1 min and rinsed with distilled water three times. In a laminar flow cabinet, the seeds were dipped in 70% (v/v) ethanol for 1 minute and blotted with sterile tissue papers. The sterile seeds were then cultured on MS basal medium.

3.2.2 Effects of Different Temperatures (18, 24 and 30±1ºC) on *In Vivo* and *In Vitro* Seeds Germination

Finally, the seeds were germinated under three different temperatures that mimic the temperate countries conditions (18ºC-autumn, 24ºC-summer) and 30ºC (tropical temperature), *in vivo* (black or garden soil) and *in vitro* (on MS media with and without $ADSO_4$). Germinations of both species were observed for 30 days.

3.2.3 Effects of $ADSO_4$ on Seeds Germination and Explants (Root, Stem and Flower Bud Growth)

Germination was carried out on MS basal media in order to obtain normal growth of *C. ternatea* L. and *O. viciifolia* Scop. under different temperatures (18ºC, 24ºC and 30ºC). While intact explants (flower bud) of *C. ternatea* L.were sterilized using the same method for seeds sterilization, but with short duration (30 seconds for each disinfectant). However, flower bud explants of *O. viciifolia* Scop. were not available from *in vivo* grown plant. adenine hemisulfate ($ADSO_4$) powder (0.1 g) was dissolved gradually in 100 ml distilled water (similar as preparation of stock

hormones) and added to Murashige and Skoog (MS) basal media with various concentrations (0-100.0 mg/L $ADSO_4$). The MS (Duchefa) media supplemented with 30 g/L sucrose (System) and 8 g/L agar (Oxoid). The pH was adjusted to 5.8 by adding 0.1 M of either sodium hydroxide (NaOH) or hydrochloric acid (HCl). Finally, the MS media was autoclaved at 104 kPa (15 Psi^2) at 121°C for 20 minutes. The sterilised media was dispensed into 1/3 of 60 ml sterile containers. Thus, 2-week-old root and stem explants were cultured on MS media supplemented with $ADSO_4$ (0-100 mg/L), NAA (1-3 mg/L), BAP, 2,4-D (1-3 mg/L) and KIN (1-3 mg/L). The treatments of $ADSO_4$ in the culture media as listed below;

1. MS basal media (control)
2. MS + 20.0 mg/l $ADSO_4$
3. MS + 40.0 mg/l $ADSO_4$
4. MS + 60.0 mg/l $ADSO_4$
5. MS + 80.0 mg/l $ADSO_4$
6. MS + 100.0 mg/l $ADSO_4$

3.2.4 Effects of NAA, BAP, 2,4-D and KIN (1.0-3.0 mg/L) on Cultures of *Clitoria ternatea* L. and *Onobrychis viciifolia* Scop.

Plant growth regulators (0.1 g) including NAA, BAP, 2,4-D and KIN were dissolved gradually in a few drops of sodium hyroxide (NaOH), then added with 100 ml distilled water, before pipetted to MS media with various concentrations (0-3.0 mg/L). After 2 weeks of germination, the root and stem explants of the aseptic seedlings were excised into small pieces (5 mm^2) and cultured on MS media with different combinations and concentrations of hormones ($ADSO_4$, NAA, BAP, 2,4-D and KIN). Thirty replicates for each treatment were used. All cultures were incubated in a culture room at 24±1°C, with a 16 hour photoperiod and 1000 lux of light intensity.

The media used as listed below;

1. MS basal media + 40.0 mg/L $ADSO_4$ (control)
2. MS + 40.0 mg/L $ADSO_4$ + 1.0 mg/l NAA
3. MS + 40.0 mg/L $ADSO_4$ + 2.0 mg/l NAA
4. MS + 40.0 mg/L $ADSO_4$ + 3.0 mg/l NAA
5. MS + 40.0 mg/L $ADSO_4$ + 1.0 mg/l BAP
6. MS + 40.0 mg/L $ADSO_4$ + 2.0 mg/l BAP
7. MS + 40.0 mg/L $ADSO_4$ + 3.0 mg/l BAP
8. MS + 40.0 mg/L $ADSO_4$ + 1.0 mg/l 2,4-D
9. MS + 40.0 mg/L $ADSO_4$ + 2.0 mg/l 2,4-D
10. MS + 40.0 mg/L $ADSO_4$ + 3.0 mg/l 2,4-D
11. MS + 40.0 mg/L $ADSO_4$ + 1.0 mg/l KIN
12. MS + 40.0 mg/L $ADSO_4$ + 2.0 mg/l KIN
13. MS + 40.0 mg/L $ADSO_4$ + 3.0 mg/l KIN
14. MS + 40.0 mg/L $ADSO_4$ + 1.0 mg/l NAA + 1.0 mg/l BAP
15. MS + 40.0 mg/L $ADSO_4$ + 2.0 mg/l NAA + 1.0 mg/l BAP
16. MS + 40.0 mg/L $ADSO_4$ + 3.0 mg/l NAA + 1.0 mg/l BAP
17. MS + 40.0 mg/L $ADSO_4$ + 1.0 mg/l NAA + 2.0 mg/l BAP
18. MS + 40.0 mg/L $ADSO_4$ + 2.0 mg/l NAA + 2.0 mg/l BAP
19. MS + 40.0 mg/L $ADSO_4$ + 3.0 mg/l NAA + 2.0 mg/l BAP
20. MS + 40.0 mg/L $ADSO_4$ + 1.0 mg/l NAA + 3.0 mg/l BAP
21. MS + 40.0 mg/L $ADSO_4$ + 2.0 mg/l NAA + 3.0 mg/l BAP
22. MS + 40.0 mg/L $ADSO_4$ +3.0 mg/l NAA + 3.0 mg/l BAP
23. MS + 40.0 mg/L $ADSO_4$ + 1.0 mg/l 2,4-D + 1.0 mg/l KIN
24. MS + 40.0 mg/L $ADSO_4$ + 2.0 mg/l 2,4-D + 1.0 mg/l KIN
25. MS + 40.0 mg/L $ADSO_4$ + 3.0 mg/l 2,4-D +1.0 mg/l KIN
26. MS + 40.0 mg/L $ADSO_4$ + 1.0 mg/l 2,4-D + 2.0 mg/l KIN

27. MS + 40.0 mg/L $ADSO_4$ + 2.0 mg/l 2,4-D + 2.0 mg/l KIN

28. MS + 40.0 mg/L $ADSO_4$ + 3.0 mg/l 2,4-D + 2.0 mg/l KIN

29. MS + 40.0 mg/L $ADSO_4$ + 1.0 mg/l 2,4-D + 3.0 mg/l KIN

30. MS + 40.0 mg/L $ADSO_4$ + 2.0 mg/l 2,4-D + 3.0 mg/l KIN

31. MS + 40.0 mg/L $ADSO_4$ + 3.0 mg/l 2,4-D + 3.0 mg/l KIN

3.2.5 Data Analysis

All experiments were conducted using a completely randomized design. Data collected were statistical analyzed using Duncan's Multiple Range Test (DMRT). Mean with different letters in the same column differ significantly at $p<0.05$.

3.3 RESULTS

3.3.1 Effects of Different temperatures (18, 24 and 30±1ºC) on *In Vivo* and *In Vitro* Seeds Germination

At the early stage of experiments, seeds of *C. ternatea* L. and *O. viciifolia* Scop. were germinated under three different temperatures (18, 24 and 30ºC). Based on the results obtained, both species germinated well under 24±1ºC with the highest stem for *C. ternatea* L. and *O. viciifolia* Scop, grown *in vivo* (black garden soil) and *in vitro* (MS basal media). Based on Table 3.1 and Figure 3.1, generally, all tested temperatures gave positive growth response in 1-month-old *C. ternatea* L., whereby was slightly higher in *in vitro* (13.15±1.15 cm) compared to *in vivo* (11.20±1.95 cm), at 24±1ºC. Under *in vivo* condition, there was 100% germination with the highest stem height was obtained under 24±1ºC (11.20±1.95). This followed by slightly shorter plant at 30±1ºC (10.60±1.21 cm), as well as at 18±1ºC (7.85±0.89). However, *in vitro* seeds germination only responded at 24±1ºC and 18±1ºC with 13.15±1.15 cm and 12.80±0.97 cm, respectively. No germination was obtained at 30±1ºC, *in vitro.*

Table 3.1 : *In vivo* and *in vitro* seeds germination at different temperatures (18, 24 and 30±1ºC) of *Clitoria ternatea* L. (tropical legume) after 4 weeks.

Temperature (ºC)	*In vivo* (soil)		*In vitro* (MS basal)	
	Germination rate (%)	Mean stem height, cm	Germination rate (%)	Mean stem height, cm
18.0 ± 1ºC	100.0 ± 0.0a	7.85 ± 0.89c	100.0 ± 0.0a	12.80 ± 0.97b
24.0 ± 1ºC	**100.0 ± 0.0a**	**11.20 ± 1.95a**	**100.0 ± 0.0a**	**13.15 ± 1.15a**
30.0 ± 1ºC	100.0 ± 0.0a	10.60 ± 1.21b	NR	NR

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). Replicates, n=30. The bold data represent the optimum value.

Table 3.2 and Figure 3.2 showed 100% seeds germination of *O. viciifolia* Scop. at 18±1ºC and 24±1ºC, grown *in vivo* and *in vitro*. Under *in vivo* condition, the highest plant (8.45±0.83 cm) was obtained at 24±1ºC, which was slightly lower as compared to *in vitro* grown plant (9.15±2.13 cm). Similar to *C. ternatea* L., no germination obtained at 30±1ºC, *in vitro.* Overall, high temperature (30±1ºC) gave

negative respond to *in vitro* germination for both perennial legume species (*C. ternatea* L. and *O. viciifolia* Scop.). In terms of plant height, the growth of *C. ternatea* L. (7.85-13.15 cm) was better than *O. viciifolia* Scop. (6.50-9.15 cm) after one month germination on MS basal media, at three different temperatures which mimic to four seasons in temperate countries (18±1ºC-autumn, 24±1ºC summer) and tropical countries (30±1ºC). Therefore, *C. ternatea* L. was capable to germinate and grow well in low and high temperature in both temperate and tropical countries, whilst *O. viciifolia* Scop. only can survive at 18±1ºC and 24±1ºC. Moreover, low temperatures gave dark green leaves quality compared to high temperatures (light green leaves) for *C. ternatea* L. and *O. viciifolia* Scop.

Table 3.2 : *In vivo* and *in vitro* seeds germination of *Onobrychis viciifolia* Scop. (temperate legume) under different temperatures (18, 24 and 30±1ºC), after 4 weeks.

Temperature (ºC)	*In vivo* (soil)		*In vitro* (MS basal)	
	Germination rate (%)	Mean stem height, cm	Germination rate (%)	Mean stem height, cm
18.0 ± 1ºC	100.0 ± 0.0a	6.50 ± 1.53b	100.0 ± 0.0a	5.65 ± 2.13b
24.0 ± 1ºC	**100.0 ± 0.0a**	**8.45 ± 0.83a**	**100.0 ± 0.0a**	**9.15 ±1.35a**
30.0 ± 1ºC	66.7 ± 8.8b	4.20 ± 0.64c	NR	NR

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). Replicates, n=30. The bold data represent the optimum value.

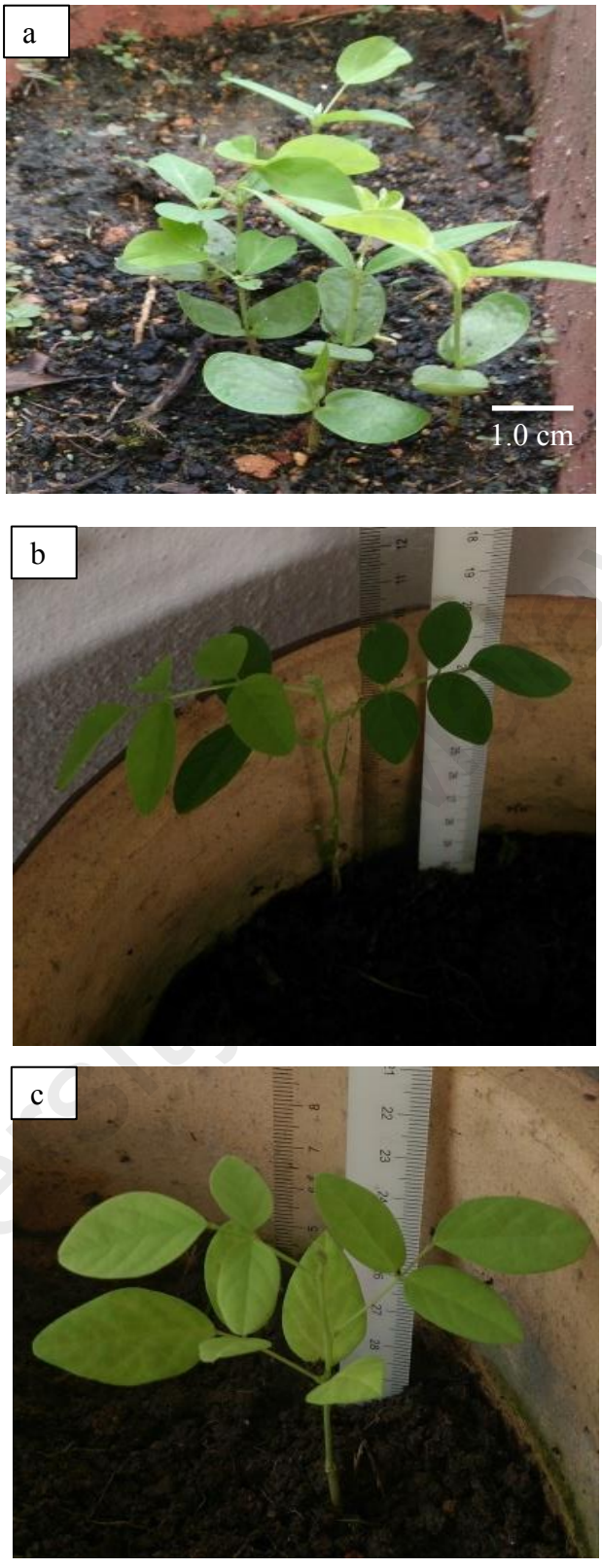


Figure 3.1 : Different height and colours of stem and leaves of *Clitoria ternatea* L. under different temperatures after one month of germination. (a) Stunted stem, darkest green leaves, at 18±1ºC. (b) Highest stem, darker green leaves, at 24 ± 1ºC. (b) Higher stem and light green leaves, at 30 ± 1ºC.

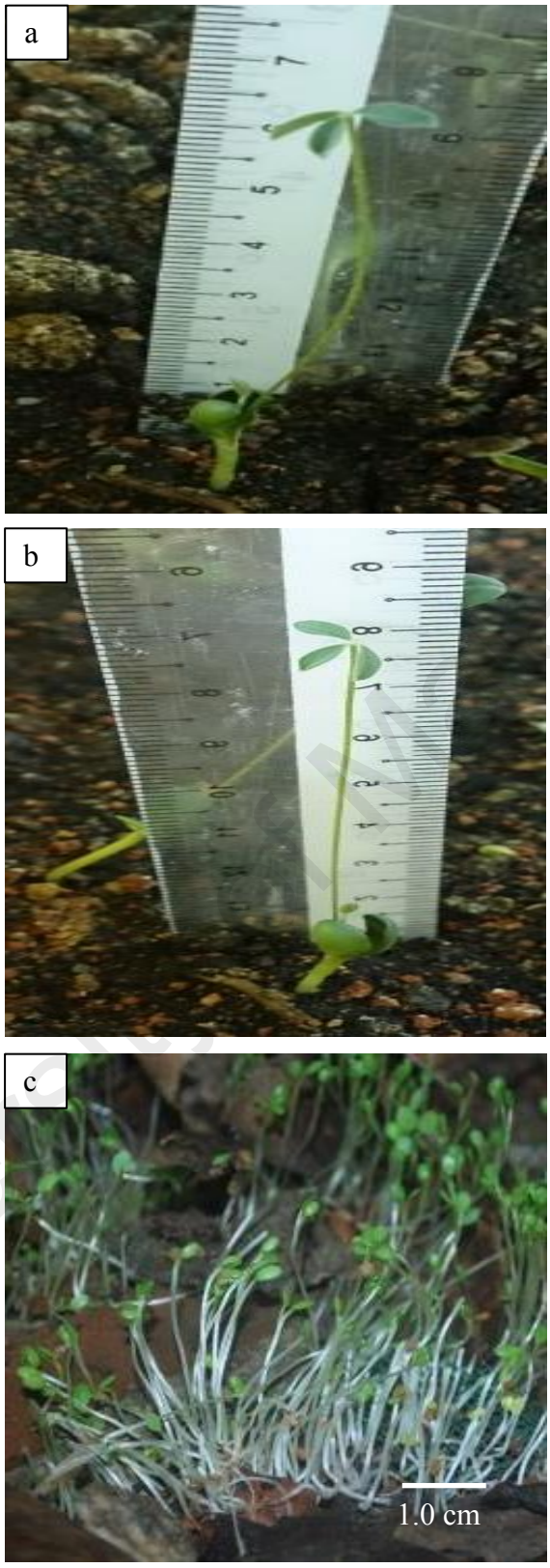


Figure 3.2 : Different height and colours of stem and leaves grown at different temperatures of 1-month-old of *Onobrychis viciifolia* Scop. (a) Higher stem and light green leaves, at 18±1ºC. (b) Highest stem, darker green leaves, at 24 ± 1ºC. (b) Only sprouting seeds with green plumule for 3-4 weeks, at 30 ± 1ºC.

3.3.2 Effects of adenine hemisulfate ($ADSO_4$) in MS Media on Growth Performances

Initially, both *C. ternatea* L. and *O. viciifolia* Scop managed to germinate and grow in *in vitro* condition at 24±1ºC cultured on MS basal media (without hormone). Then, both species were germinated on MS media added with different concentrations of $ADSO_4$ (0-100 mg/L). Table 3.3 showed the different response of *in vitro* growth performance (normal and abnormal), as well as formation of white callus on stem and roots explants. $ADSO_4$, at low concentrations (0-60 mg/L) resulted in normal growth performances (development of shoots, stem, leaves and roots), while, at high concentrations (80-100 mg/L), the plants grew abnormally for both *C. ternatea* L. (Figure 3.3) and *O. viciifolia* Scop. (Figure 3.4). Generally, *C. ternatea* L. responded positively to all treatments with white callus formation from root and stem explants, but at different culture duration. As control, *C. ternatea* L. cultured on MS *b*asal media, produced white callus from stem, followed by rooting after 6 months, while *O. viciifolia* Scop. grew normally. However, when MS supplemented with 40.0 mg/L $ADSO_4$, white callus formation of *C. ternatea* L. grown rapidly and vigorously at taproot (in 10 days) and followed by stem (in 30 days) explants. Normal growth was observed on MS supplemented with 0-20 mg/L $ADSO_4$, while abnormal growth was observed at 80-100 mg/L $ADSO_4$ with different morphology whereby the leaves were stunted and a lot of callus formed on root explants (after 3-6 weeks). However, under the same treatment (MS supplemented with 40 mg/L $ADSO_4$), in *O. viciifolia* Scop., white callus formation was observed but only at taproot (in 20 days), while stem explants grew normally. Normal growth was observed on MS supplemented with 0-20 mg/L $ADSO_4$, while abnormal growth at 60-100 mg/L $ADSO_4$ with formation of swollen secondary roots after 2-3 months.

Table 3.3 : The effects of adenine hemisulfate ($ADSO_4$) on *Clitoria ternatea* L. (tropical legume) and *Onobrychis viciifolia* Scop. (temperate legume).

$MS+ADSO_4$ (mg/L)	Observations	
	Clitoria ternatea L.	*Onobrychis viciifolia* Scop.
MS+0	Normal growth. Formation of white callus on taproot and stems after 6 months.	Normal growth.
MS+20	Normal growth. Formation of white callus on taproot and stems after 4 months.	Normal growth.
MS+40	**Taproot and stems produced white callus, in 10 and 30 days, respectively.**	**Only taproot produced white callus in 20 days.**
MS+60	Normal growth. Only taproot produced callus after 2 months.	Abnormal growth with swollen of whole part of root (taproot and secondary root) after 3 months.
MS+80	After 6 weeks, abnormal growth occured. Roots produced a lot of callus, while leaves became stunted. No callus formation on stem.	Abnormal growth with swollen of whole part of root (taproot and secondary root) after 2 months.
MS+100	After 4 weeks, abnormal growth occured. Roots produced a lot of callus, while leaves became stunted. No callus formation on stem.	Abnormal growth with swollen of whole part of root (taproot and secondary root) after 2 months.

Values are in Mean ± Standard Error (SE). 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). Replicates, n=30. The bold data represent the optimum value.

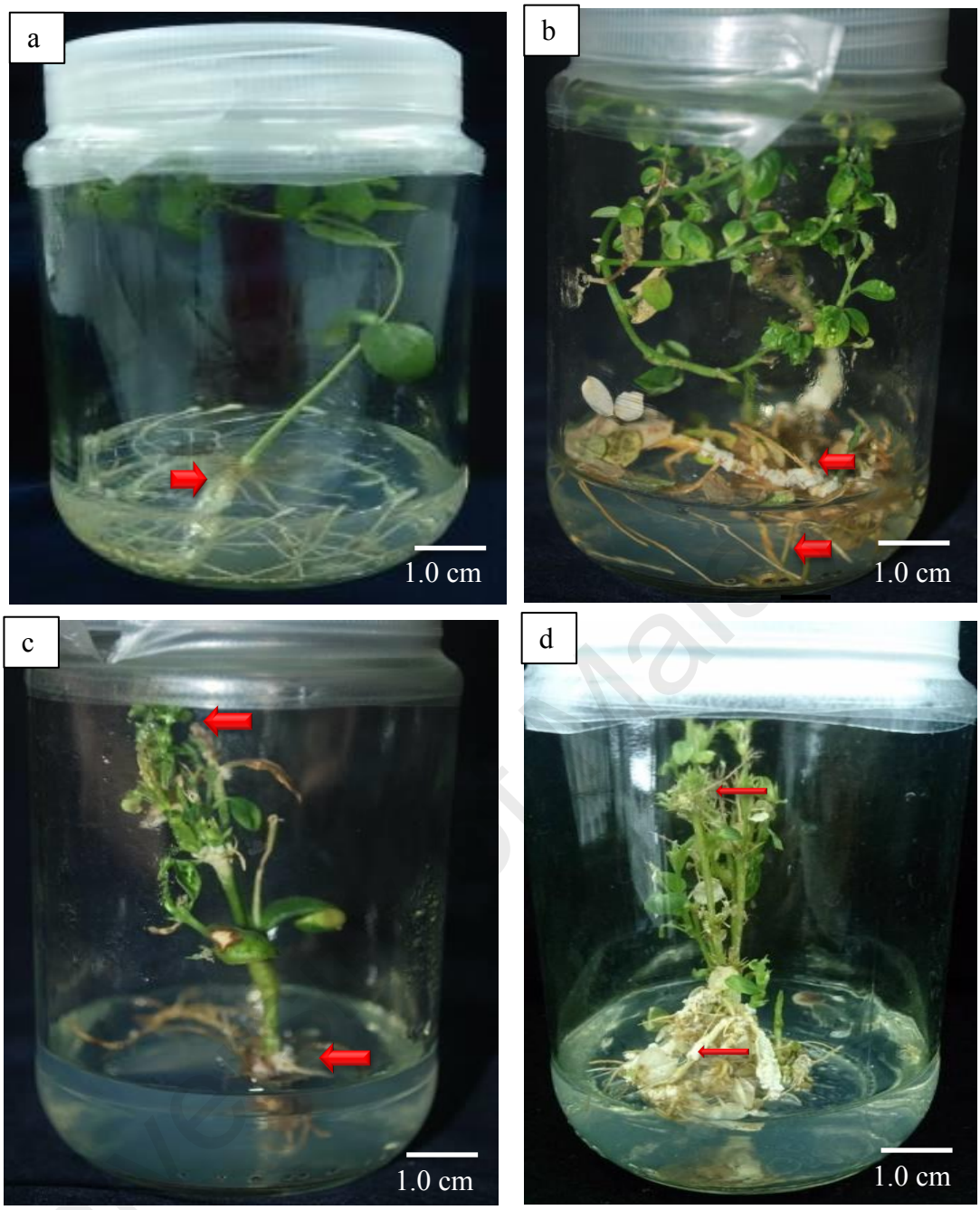


Figure 3.3 : Effects of $ADSO_4$ on in vitro germination of *Clitoria ternatea* L. on MS media at 24.0±1.0ºC. (a) White callus formation at taproot on MS+40.0 mg/L $ADSO_4$, after 10 days. (b) Vigorous growth and white callus formation at taproot and stem on MS+40.0 mg/L $ADSO_4$, after 30 days. (c) Abnormal growth of roots and leaves on MS+80.0 mg/L $ADSO_4$. (d) Abnormal growth of roots and leaves on MS+100.0 mg/L $ADSO_4$.

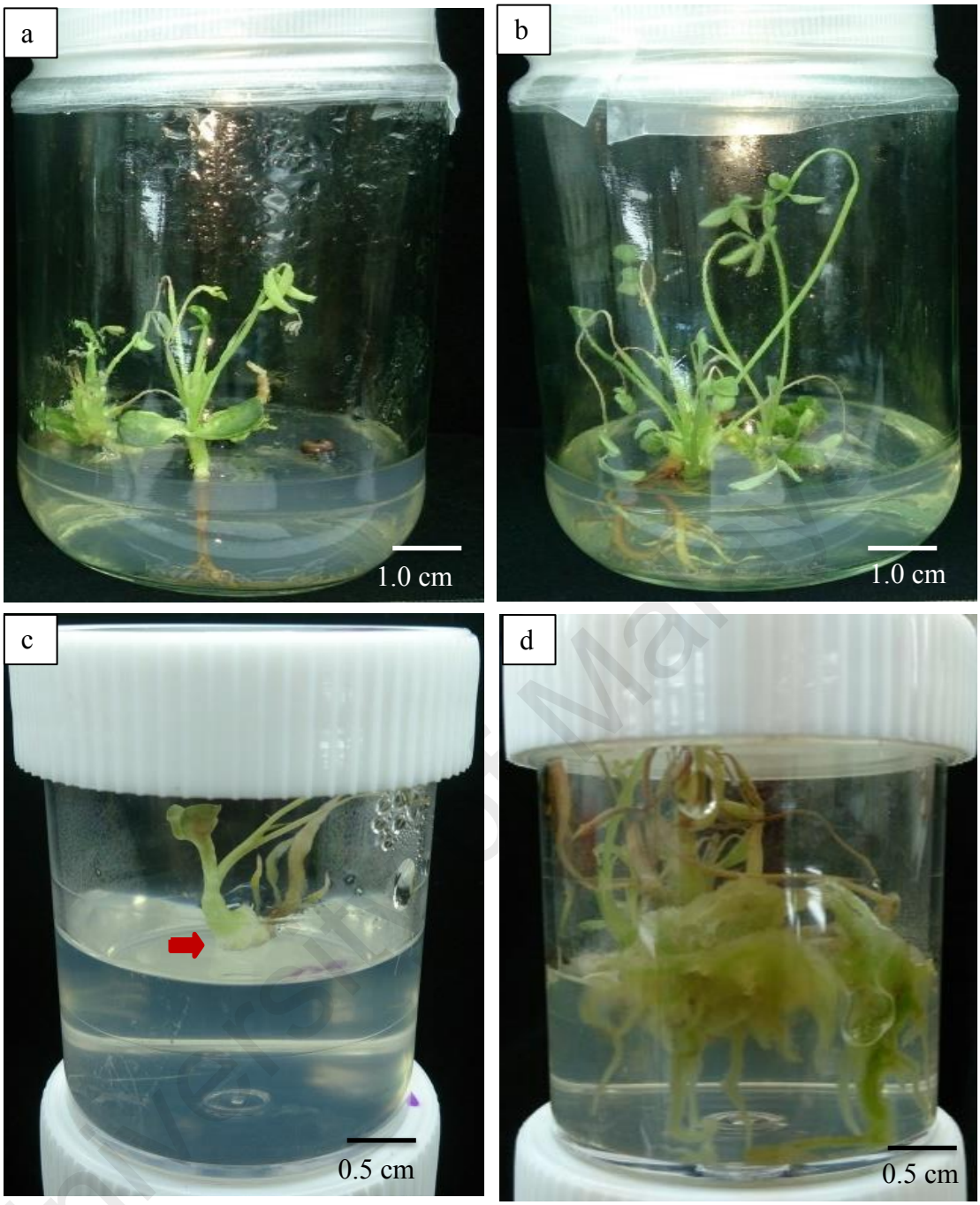


Figure 3.4 : Effects of $ADSO_4$ on *in vitro* germination of *Onobrychis viciifolia* Scop. On MS media at 24.0±1.0ºC. (a) Normal germination on MS basal. (b) Vigorous growth on MS+40.0 mg/L $ADSO_4$. (c) Callus formation at taproot on MS+40.0 mg/L $ADSO_4$, after 10 days. (d) Abnormal growth of swollen whole roots on MS+80.0 mg/L $ADSO_4$ and MS+100.0 mg/L $ADSO_4$.

3.3.3 Effect of NAA, BAP, 2,4-D and KIN to $ADSO_4$-supplemented MS Media

The results (Figure 3.5) revealed the responses of *C. ternatea* L (root, stem and flower bud explants) and *O. viciifolia* Scop. (root and stem explants) cultured on $ADSO_4$-supplemented MS media added with different concentrations of NAA, BAP, 2,4-D and KIN, applied singly and in combinations. Initially, 10-day-old explants (root and stem) cultured on MS media added with 40 mg/L $ADSO_4$ (control), were subcultured to $ADSO_4$-supplemented MS media supplemented with NAA, BAP, 2,4-D and KIN (0-3 mg/L, respectively), as well as for intact explant of *C. ternatea* L. (flower bud) using the same method as seeds sterilization but with shorter duration of sterilisation (30 seconds each). Unfortunately, for *O. viciifolia* Scop. no flower buds were obtained in Malaysia, thus, similar test could not be carried out.

Based on the results (Table 3.4, 3.5, 3.6 and 3.7), all explants produced callus (100%) for all treatments for both species, and only flower bud explant remain as callus after 20 weeks. Root explant was found as the optimum explant for both *C. ternatea* L. and *O. viciifolia* Scop. In Table 3.4 (treatment with NAA and BAP) and Table 3.5 (treatments with 2,4-D and KIN) for *C. ternatea* L., root was found to be the best explant for multiple shoots formation. The highest shoots formation per explant (15.33±0.51) was obtained from MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$ and 2.0 mg/L KIN, then followed by slightly lower (12.03±0.12) on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$, 2.0 mg/L NAA and 1.0 mg/L BAP. However, the highest roots per explant (8.72±1.27) was obtained on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$, 2.0 mg/L NAA and 1.0 mg/L BAP, as compared with MS medium supplemented with 40.0 mg/L $ADSO_4$ and 2.0 mg/L KIN (2.61±0.77). Moreover, stem explant produced the highest number of multiple shoots (5.47±0.10) on MS medium supplemented with combination of 40.0 mg/L $ADSO_4$,

2.0 mg/L NAA and 3.0 mg/L BAP, followed by MS medium supplemented with 40.0 mg/L $ADSO_4$ and 1.0 mg/L KIN (2.57±0.09). Therefore, the results suggested that multiple shoots formation from root explant (Figure 3.5) of *C. ternatea* L. responded better when supplemented with single KIN, however, the root formation was better under combination treatments of NAA and BAP. Stem explants only managed to produce half as compared with root explant. However, in this experiment, the chosen optimum medium for *C. ternatea* L. regeneration from root explant was MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$, 2.0 mg/L NAA and 1.0 mg/L BAP, for formation of both shoots and roots.

Table 3.4 : The effects of different concentrations and combinations of NAA and BAP on root, stem and flower bud explants of *Clitoria ternatea* L. cultured on MS media for 20 weeks. The cultures were maintained at 24±1ºC with 16 hours light and 8 hours dark, under 1000 lux intensity.

MS+40 mg/L $ADSO_4$		Explants	Mean number of shoots per explant	Mean number of roots per explant
NAA	BAP			
0	0	Root	3.90±0.15d	0
		Stem	0	0
		Flower	0	0
1.0		Root	0	0
		Stem	0	0
		Flower	0	0
2.0		Root	7.60±0.09b	0
		Stem	0	0
		Flower	0	0
3.0		Root	0	0
		Stem	0	0
		Flower	0	0
	1.0	Root	0	0
		Stem	0	0
		Flower	0	0
	2.0	Root	0	0
		Stem	0	0
		Flower	0	0
	3.0	Root	0	0
		Stem	0	0
		Flower	0	0
1.0	1.0	Root	5.47±0.09c	0
		Stem	0	0
		Flower	0	0
2.0	**1.0**	**Root**	**12.03±0.12a**	**8.72±1.27a**
		Stem	0	0
		Flower	0	0
3.0	1.0	Root	0	0
		Stem	0	0
		Flower	0	0
1.0	2.0	Root	0	0
		Stem	3.10±0.12b	0
		Flower	0	0
2.0	2.0	Root	0	0
		Stem	0	0
		Flower	0	0
3.0	2.0	Root	0	0
		Stem	0	0
		Flower	0	0
1.0	3.0	Root	0	0
		Stem	5.47±0.10a	0
		Flower	0	0

'**Table 3.4**, continued'

2.0	3.0	Root	0	0
		Stem	0	0
		Flower	0	0
3.0	3.0	Root	0	0
		Stem	0	0
		Flower	0	0

Values are Means±Standard Error (SE). 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). Replicates, n=30. The bold value is represented the best result foreach explant (root, stem and flower bud).

Table 3.5 : The effects of different concentrations and combinations of 2,4-D and KIN on root, stem and flower bud explants of *Clitoria ternatea* L. cultured on MS media for 20 weeks. The cultures were maintained at 24±1ºC with 16 hours light and 8 hours dark, under 1000 lux intensity.

MS+40 mg/L $ADSO_4$		Explants	Mean number of shoots per explant	Mean number of roots per explant
2,4-D	KIN			
0	0	Root	3.90±0.15d	0
		Stem	0	0
		Flower	0	0
1.0		Root	0	0
		Stem	0	0
		Flower	0	0
2.0		Root	0	0
		Stem	0	0
		Flower	0	0
3.0		Root	0	0
		Stem	0	0
		Flower	0	0
	1.0	Root	0	0
		Stem	2.57±0.09a	0
		Flower	0	0
	2.0	**Root**	**15.33±0.51a**	**2.67±0.77a**
		Stem	1.43±0.09b	0
		Flower	0	0
	3.0	Root	0	0
		Stem	0	0
		Flower	0	0
1.0	1.0	Root	13.23±0.11b	0
		Stem	0	0
		Flower	0	0
2.0	1.0	Root	0	0
		Stem	0	0
		Flower	0	0
3.0	1.0	Root	0	0
		Stem	0	0
		Flower	0	0
1.0	2.0	Root	0	0
		Stem	0	0
		Flower	0	0
2.0	2.0	Root	0	0
		Stem	0	0
		Flower	0	0
3.0	2.0	Root	0	0
		Stem	0	0
		Flower	0	0
1.0	3.0	Root	0	0
		Stem	5.47±0.10a	0
		Flower	0	0

‘**Table 3.5**, continued’

2.0	3.0	Root	0	0
		Stem	0	0
		Flower	0	0
3.0	3.0	Root	0	0
		Stem	0	0
		Flower	0	0

Values are Means±Standard Error (SE). 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). Replicates, n=30. The bold value is represented the best result foreach explant (root, stem and flower bud).

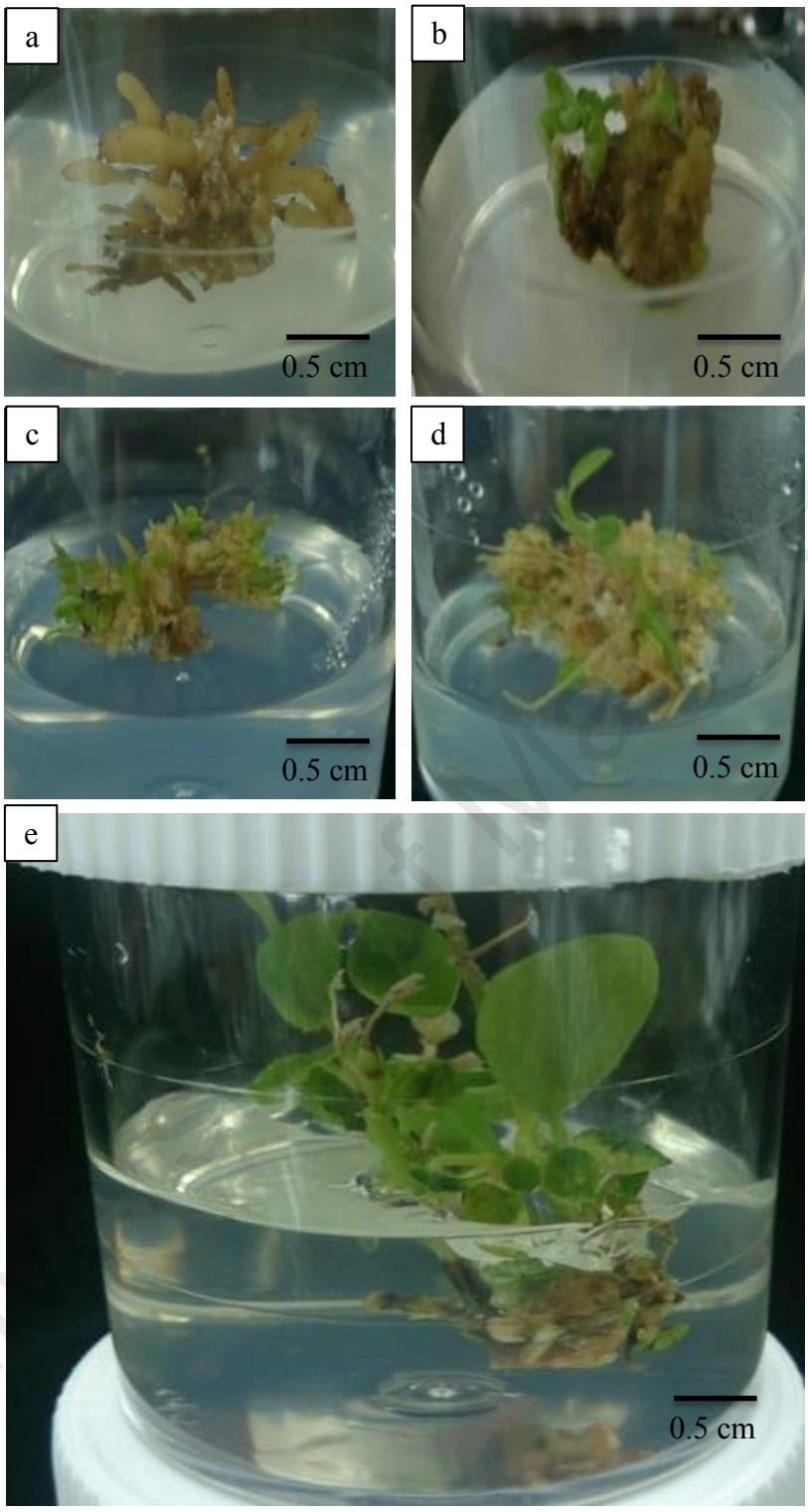


Figure 3.5 : *In vitro* regeneration through callus formation of *Clitoria ternatea* L. from root explants cultured on MS media supplemented with combinations of 40.0 mg/L $ADSO_4$, 2.0 mg/L NAA and 1.0 mg/L BAP. (a) Root explant. (b) Somatic embryogenesis. (c) Multiple shoots formation. (d) Roots formation. (e) Shoots elongation.

From Tables 3.6 and 3.7, the highest shoots (17.97±0.09 mm) and roots (4.11±0.42 mm) formation of *O. viciifolia* Scop. were obtained from root explants (Figure 3.6) cultured on the same MS medium (MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$, 1.0 mg/L 2,4-D and 3.0 KIN), then, followed by 9.07±0.14 shoots per explant cultured on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$, 1.0 mg/L NAA and 1.0 mg/L BAP). While, stem explants managed to produce the highest shoots (3.97±0.13 number of shoots per explant) when cultured on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$, 2.0 mg/L NAA and 1.0 mg/L BAP, followed by 2.33±0.09 number of shoots per explant on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$ and 2.0 mg/L KIN.

Table 3.6 : The effects of different concentrations and combinations of NAA and BAP on root and stem explants of *Onobrychis viciifolia* Scop. cultured on MS media for 20 weeks. The cultures were maintained at 24±1ºC with 16 hours light and 8 hours dark, under 1000 lux intensity.

MS+40 mg/L $ADSO_4$		Explants	Mean number of shoots per explant	Mean number of roots per explant
NAA	BAP			
0	0	Root	7.40±0.09d	0
		Stem	0	0
1.0		Root	6.30±0.09e	0
		Stem	0	0
2.0		Root	0	0
		Stem	0	0
3.0		Root	0	0
		Stem	0	0
	1.0	Root	8.77±0.08b	0
		Stem	0	0
	2.0	Root	4.70±0.09f	0
		Stem	0	0
	3.0	Root	0	0
		Stem	0	0
1.0	**1.0**	**Root**	**9.07±0.14a**	0
		Stem	0	0
2.0	1.0	Root	7.07±0.09c	0
		Stem	2.93±0.12g	0
3.0	1.0	Root	0	0
		Stem	0	0
1.0	2.0	Root	0	0
		Stem	0	0
2.0	2.0	Root	0	0
		Stem	2.70±0.09b	0
3.0	2.0	Root	0	0
		Stem	0	0
1.0	3.0	Root	0	0
		Stem	5.47±0.10a	0
2.0	3.0	Root	0	0
		Stem	0	0
3.0	3.0	Root	0	0
		Stem	0	0

Values are Means±Standard Error (SE). 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). Replicates, n=30. The bold value is represented the best result foreach explant (root and stem).

Table 3.7 : The effects of different concentrations and combinations of NAA and BAP on root and stem explants of *Onobrychis viciifolia* Scop. cultured on MS media for 20 weeks. The cultures were maintained at 24±1ºC with 16 hours light and 8 hours dark, under 1000 lux intensity.

MS+40 mg/L $ADSO_4$		Explants	Mean number of shoots per explant	Mean number of roots per explant
2,4-D	KIN			
0	0	Root	7.40±0.09c	0
		Stem	0	0
1.0		Root	6.30±0.85d	0
		Stem	0	0
2.0		Root	0	0
		Stem	0	0
3.0		Root	0	0
		Stem	0	0
	1.0	Root	0	0
		Stem	0	0
	2.0	Root	0	0
		Stem	2.33±0.09a	0
	3.0	Root	0	0
		Stem	0	0
1.0	1.0	Root	14.40±0.10b	0
		Stem	0	0
2.0	1.0	Root	0	0
		Stem	0	0
3.0	1.0	Root	0	0
		Stem	0	0
1.0	2.0	Root	0	0
		Stem	1.47±0.09b	0
2.0	2.0	Root	0	0
		Stem	0	0
3.0	2.0	Root	0	0
		Stem	0	0
1.0	**3.0**	**Root**	**17.97±0.09a**	**4.11±0.42a**
		Stem	0	0
2.0	3.0	Root	0	0
		Stem	0	0
3.0	3.0	Root	0	0
		Stem	0	0

Values are Means±Standard Error (SE). 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). Replicates, n=30. The bold value is represented the best result foreach explant (root and stem).

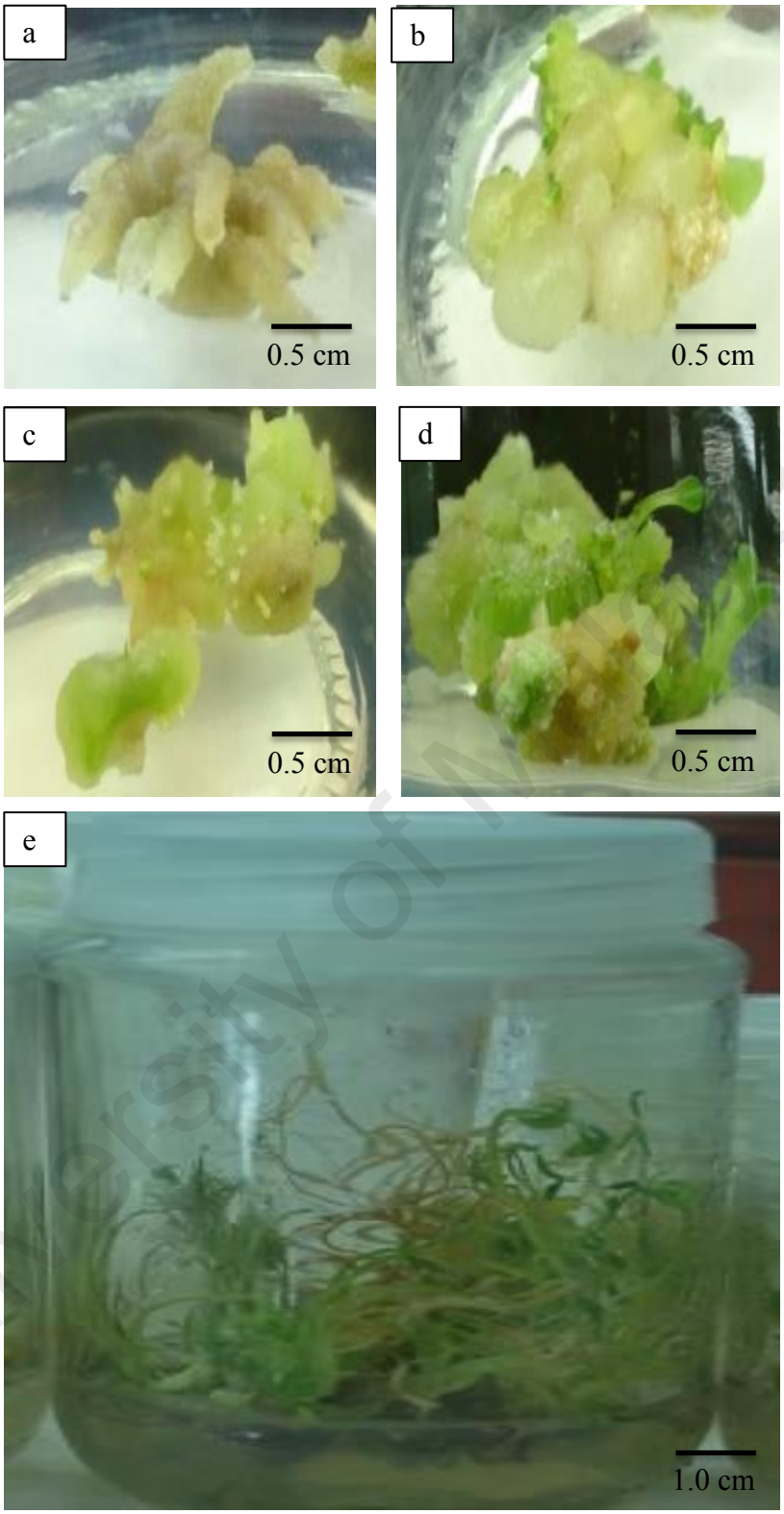


Figure 3.6 : *In vitro* regeneration through callus formation of *Onobrychis viciifolia* Scop. from root explants cultured on MS medium supplemented with combnations of 40.0 mg/L $ADSO_4$, 1.0 mg/L NAA and 1.0 mg/L BAP. (a) Root explant. (b) Globular stage of callus. (c) Somatic embryos. (d) Multiple shoots formation. (e) Plantlets or regenerated plants.

3.4 SUMMARY OF RESULTS

1. The best temperature for seeds germination for *C. ternatea* L. and *O. viciifolia* Scop.) was at 24±1ºC, grown *in vivo* and *in vitro*.

2. White callus formation of *C. ternatea* L. was rapidly and vigorously obtained from seed germination on MS media supplemented with 40.0 mg/L $ADSO_4$ at taproot (10 days) and followed by stem (30 days) explants.

3. *O. viciifolia* Scop. responded to MS media supplemented with 40.0 mg/L $ADSO_4$ which produced white callus only at taproot (in 20 days).

4. The highest shoots formation (15.33±0.51) of *C. ternatea* L. was obtained from root explant that were cultured on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$ and 2.0 mg/L KIN, after 20 weeks.

5. The highest root formation (8.72±1.27) for *C. ternatea* L. was obtained from root explants that were cultured on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$, 2.0 mg/L NAA and 1.0 mg/L BAP, after 20 weeks, with slightly lower shoots formation (12.03±0.12) (chosen as optimum media).

6. The highest shoots (17.97±0.09) and roots (4.11±0.42) formation for *O. viciifolia* Scop. was obtained from root explant cultured on the same media (MS supplemented with 40.0 mg/L $ADSO_4$, 1.0 mg/L 2,4-D and 3.0 mg/L KIN), after 20 weeks.

7. Generally, the best explant for both species was root explant (2-3 weeks old) for shoots and roots formation, however, *C. ternatea* L. was more responsive to MS media supplemented with combinations of 40.0 mg/L $ADSO_4$, NAA and BAP, while *O. viciifolia* Scop. responded best on MS media supplemented with combinations of 40.0 mg/L $ADSO_4$, 2,4-D and KIN. The growth of *O. viciifolia* Scop. was two times faster than *C. ternatea* L.

CHAPTER 4

CALLUS INDUCTION AND SOMATIC EMBRYOGENESIS OF *Clitoria ternatea* L. AND *Onobrychis viciifolia* Scop. FROM VARIOUS EXPLANTS (ROOT, STEM AND FLOWER BUD)

4.1 EXPERIMENTAL AIMS

Traditional production of *Clitoria ternatea* L. and *Onobrychis viciifolia* Scop. using seeds is unreliable due to poor germination and competition among the young seedlings under natural conditions. Thus, somatic embryogenesis offers an alternative and efficient mean for plant multiplication (Roberts et al., 1995). Somatic embryogenesis is a significant way for production of plants without somaclonal variation and an efficient cloning and genetic transformation (Sharp et al., 1980).

Somatic embryos are embryo-like structures which can be originating from cells other than gametes (somatic cells) without fertilization process (Parrott et al., 1988), which is genetically identical to the mother plant. Indirect somatic embryos were obtained through callus formation as compared to direct somatic embryo that influence by organized tissue (such as shoot and root). The regeneration of *C. ternatea* L. and *O. viciifolia* Scop. via somatic embryogenesis provide an excellent opportunity for rapid mass propagation. The somatic embryos undergo globular, heart, torpedo and cotyledonary shaped stages.

In vitro propagation through somatic embryogenesis is a viable alternative for a species which is difficult to regenerate by conventional methods; where populations have decreased due to over exploitation by destructive harvesting and can effectively be used to meet the growing demand for clonally uniform elite plants. Therefore, the development of micropropagation protocols for elite stocks of *C. ternatea* L. and *O. viciifolia* Scop. are urgently needed.

The objectives of this specific chapter were:

1. To establish an optimum protocol for callus induction from root, stem and flower bud explants of *C. ternatea* L. and *O. viciifolia* Scop. using different hormones and concentrations ($ADSO_4$, NAA, BAP, 2,4-D and KIN).

2. To distinguish embryogenic and non-embryogenic callus using double staining method from root, stem and flower bud explants of *C. ternatea* L. and *O. viciifolia* Scop.

3. To compare the structure, type and colour of somatic embryos in differentiation stages (globular, heart, torpedo and cotyledonary shape).

4.2 MATERIALS AND METHODS

4.2.1 Plant Materials and Culture Conditions

Root and stem explants (5.0 mm^2) of *C. ternatea* L. and *O. viciifolia* Scop. from 2-week-old aseptic seedling were cultured on $ADSO_4$-added MS media supplemented with NAA, BAP, 2,4-D and KIN (1.0-3.0 mg/L), as well as intact explant (flower bud explants were only from *C. ternatea* L.). The 30 culture replicates were maintained at 24±1 ºC, 1000 lux light intensity for 8 weeks.

4.2.2 Effects of NAA, BAP, 2,4-D and KIN on Callus Induction of Various Explants (Root, Stem and Flower Bud)

Plant growth regulators (0.1 g); NAA, BAP, 2,4-D and KIN were dissolved gradually in a few drops of sodium hyroxide (NaOH), then added with 100 ml distilled water, before pipetted to MS media with various concentrations (0-3.0 mg/L). After 2 weeks of germination, the root and stem explants were excised into small pieces (5 mm^2) and cultured on MS media with different combinations and concentration of hormones ($ADSO_4$, NAA, BAP, 2,4-D and KIN). Thirty replicates for each treatment were used. All cultures were incubated in a culture room at 24±1°C, with a 16 hour photoperiod with 1000 lux of light intensity.

The media used were as listed below;

1. MS basal media + 40.0 mg/L $ADSO_4$ (control)
2. MS + 40.0 mg/L $ADSO_4$ + 1.0 mg/l NAA
3. MS + 40.0 mg/L $ADSO_4$ + 2.0 mg/l NAA
4. MS + 40.0 mg/L $ADSO_4$ + 3.0 mg/l NAA
5. MS + 40.0 mg/L $ADSO_4$ + 1.0 mg/l BAP
6. MS + 40.0 mg/L $ADSO_4$ + 2.0 mg/l BAP
7. MS + 40.0 mg/L $ADSO_4$ + 3.0 mg/l BAP
8. MS + 40.0 mg/L $ADSO_4$ + 1.0 mg/l 2,4-D

9. MS + 40.0 mg/L $ADSO_4$ + 2.0 mg/l 2,4-D
10. MS + 40.0 mg/L $ADSO_4$ + 3.0 mg/l 2,4-D
11. MS + 40.0 mg/L $ADSO_4$ + 1.0 mg/l KIN
12. MS + 40.0 mg/L $ADSO_4$ + 2.0 mg/l KIN
13. MS + 40.0 mg/L $ADSO_4$ + 3.0 mg/l KIN
14. MS + 40.0 mg/L $ADSO_4$ + 1.0 mg/l NAA + 1.0 mg/l BAP
15. MS + 40.0 mg/L $ADSO_4$ + 2.0 mg/l NAA + 1.0 mg/l BAP
16. MS + 40.0 mg/L $ADSO_4$ + 3.0 mg/l NAA + 1.0 mg/l BAP
17. MS + 40.0 mg/L $ADSO_4$ + 1.0 mg/l NAA + 2.0 mg/l BAP
18. MS + 40.0 mg/L $ADSO_4$ + 2.0 mg/l NAA + 2.0 mg/l BAP
19. MS + 40.0 mg/L $ADSO_4$ + 3.0 mg/l NAA + 2.0 mg/l BAP
20. MS + 40.0 mg/L $ADSO_4$ + 1.0 mg/l NAA + 3.0 mg/l BAP
21. MS + 40.0 mg/L $ADSO_4$ + 2.0 mg/l NAA + 3.0 mg/l BAP
22. MS + 40.0 mg/L $ADSO_4$ +3.0 mg/l NAA + 3.0 mg/l BAP
23. MS + 40.0 mg/L $ADSO_4$ + 1.0 mg/l 2,4-D + 1.0 mg/l KIN
24. MS + 40.0 mg/L $ADSO_4$ + 2.0 mg/l 2,4-D + 1.0 mg/l KIN
25. MS + 40.0 mg/L $ADSO_4$ + 3.0 mg/l 2,4-D +1.0 mg/l KIN
26. MS + 40.0 mg/L $ADSO_4$ + 1.0 mg/l 2,4-D + 2.0 mg/l KIN
27. MS + 40.0 mg/L $ADSO_4$ + 2.0 mg/l 2,4-D + 2.0 mg/l KIN
28. MS + 40.0 mg/L $ADSO_4$ + 3.0 mg/l 2,4-D + 2.0 mg/l KIN
29. MS + 40.0 mg/L $ADSO_4$ + 1.0 mg/l 2,4-D + 3.0 mg/l KIN
30. MS + 40.0 mg/L $ADSO_4$ + 2.0 mg/l 2,4-D + 3.0 mg/l KIN
31. MS + 40.0 mg/L $ADSO_4$ + 3.0 mg/l 2,4-D + 3.0 mg/l KIN

4.2.3 Identification of Embryogenic Callus using Double Staining Method

4.2.3.1 Preparation of 2% Acetocarmine

Glacial acetic acid (45 ml) was added to 55 ml distilled water in a conical flask and added with 2.0 g of carmine. The solution was placed on a hot plate in the fume

hood and boiled gently for 5 minutes. The cooled solution was filtered using Whatman filter paper and stored at room temperature.

4.2.3.2 Preparation of 0.5% Evan's Blue

Evan's Blue powder (0.5 g) was added to 100 ml distilled water in a conical flask. The flask was then capped and swirled by hand to mix the solution.

4.2.3.3 Double Staining Technique

Callus (1 mm^2) was placed on a glass slide. A few drops of 2% acetocarmine was added to the callus. The callus was then gently divided into very small pieces in the acetocarmine using a needle. The slide was held with forceps and heated over a low flame for a few seconds. Then, the callus was washed 2 to 3 times with distilled water and all liquid was removed. Two or three drops of Evan's Blue was added to acetocarmine stained cells and after 30 seconds the stained cells were washed 2 to 3 times with water and all the water was removed. Two drops of glycerol was added to the stained cells to prevent the preparation from drying. The specimen was then observed under the image analyzer.

4.2.4 Examination of Somatic Embryo Stages

Somatic embryos (6-week-old) of *C. ternatea* L. and *O. viciifolia* Scop. were observed and compared (structure, type and colour) under image analyzer for identification of somatic embryo stages (globular, heart, torpedo and cotyledonary shape).

4.2.5 Data Analysis

All experiments were conducted using a completely randomized design. Data collected were statistical analyzed using Duncan's Multiple Range Test (DMRT). Mean with different letters in the same column differ significantly at $p<0.05$.

4.3 RESULTS

4.3.1 Determination of Embryogenic and Non-embryogenic Callus from Root, Stem and Flower Bud Explants

Table 4.1 to 4.4 show the effects of hormones added to MS media for callus induction from root, stem and flower bud explants of *C. ternatea* L. and *O. viciifolia* Scop.. The edges of explants became swollen to form callus structures, after 4 days. All callus were tested using double staining method for identification of embryogenic and non-embryogenic callus after 5 weeks. Observations were done using image analyzer (magnification 100x), whereby the embryogenic callus nuclei were stained with intense bright red with acetocarmine and non-embryogenic cells stained blue (Figure 4.1). However, when 2-week-old callus (juvenile stage) were tested, there were none embryogenic cell identified, possibly the maturation of callus influenced the staining activity. Basically, all embryogenic cells had large nuclei and dense cytoplasms. These nuclei stained intensed bright red with acetocarmine. After a few weeks, the embryogenic callus changed to shoot s formation. Somatic embryogenesis system offers an ideal process for *in vitro* production of plants.

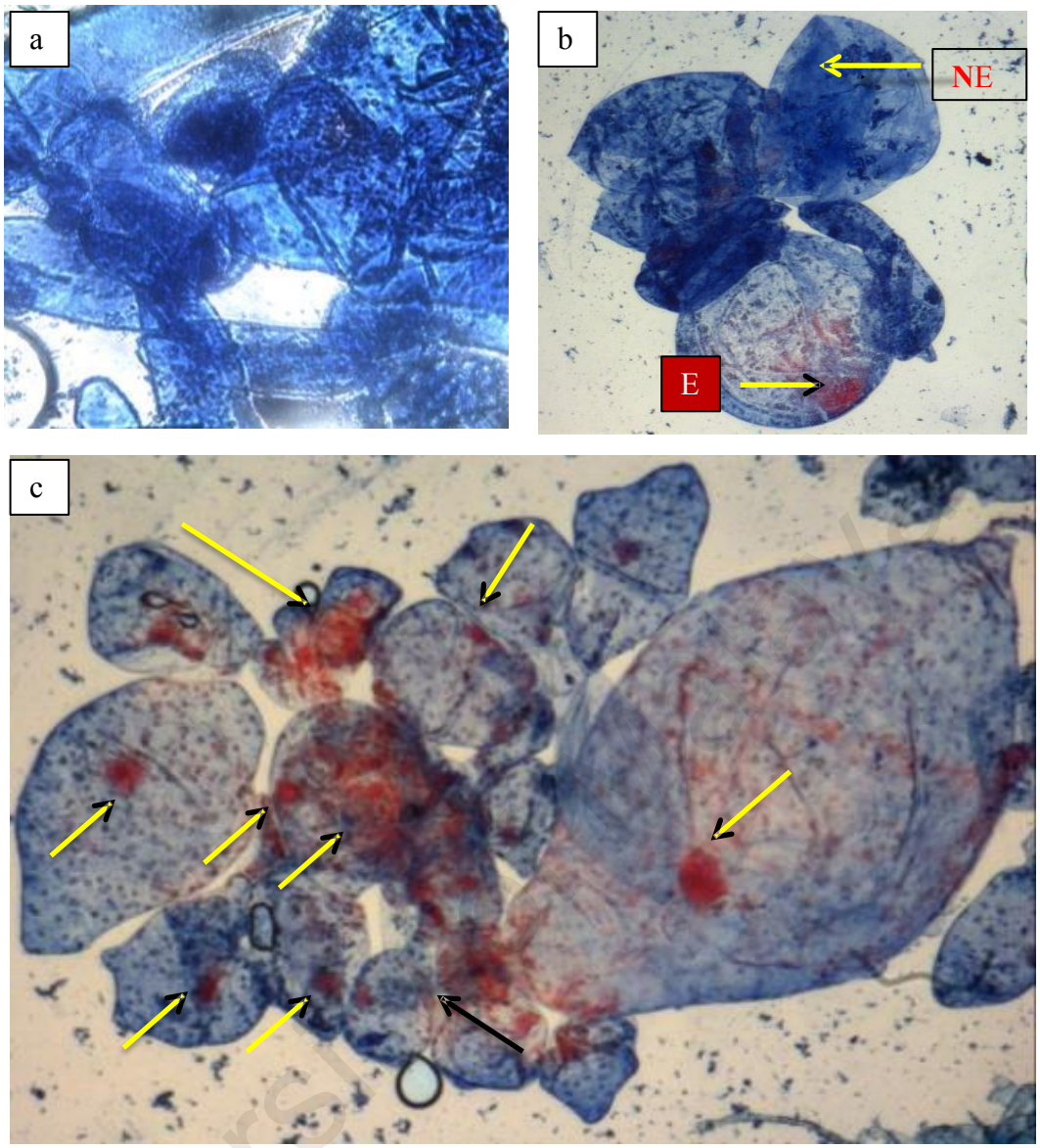


Figure 4.1: Non-embryogenic and embryogenic callus identification from root, stem and flower bud explants of *Clitoria ternatea* L. and *Onobrychis viciifolia* Scop. using double staining method observed with image analyzer at 100x magnification. (a) Non-embryogenic young callus (2-week-old) as the cells stained totally in blue colour by Evan's blue. (b) Combination of non-embryogenic callus (nucleus with blue stained) and embryogenic (nucleus with red stained. (c) Embryogenic callus identified with nucleus in red colour by acetocarmine. NE=non-embryogenic, E=embryogenic.

4.3.2 Effects of Various Hormones (NAA, BAP, 2,4-D and KIN) on Callus Induction and Somatic Embryos from Root, Stem and Flower Bud of *Clitoria ternatea* L.

Generally, all explants (root, stem and flower bud) produced callus after 2 weeks. However, based on Table 4.1 and Table 4.2, root and stem explants successfully formed dark green compact callus, embryogenic and non-embryogenic, respectively. The highest dried callus from root explant (0.43±0.02 g of embryogenic callus) was obtained on MS medium supplemented with combination of 40.0 mg/L $ADSO_4$ and 2.0 mg/L KIN.

Furthermore, stem (0.23±0.01 g non-embryogenic callus on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$ and 3.0 mg/L KIN, while, flower bud produced 0.21±0.05 g non-embryogenic callus on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$, 3.0 mg/L NAA and 3.0 mg/L BAP. The comparison of embryogenic callus induction of *C. ternatea* L. and *O. viciifolia* Scop. from root and stem explants were shown in Figure 4.2 and Figure 4.3, respectively.

Flower bud explant formed only non-embryogenic dark green compact callus with the highest dried callus (0.21±0.05 g) on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$, 3.0 mg/L NAA and 3.0 mg/L BAP, while the same explant (flower bud) responded differently on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$ and 3.0 mg/L 2,4-D which produced 0.18±0.07 g of dried friable callus (Figure 4.4).

Table 4.1 : The effects of different concentrations and combinations of NAA and BAP on callus induction from root, stem and flower bud explants of *Clitoria ternatea* L. cultured on MS media added with 40.0 mg/L $ADSO_4$ for 8 weeks. The cultures were maintained at 24±1ºC with 16 hours light and 8 hours dark (1000 lux intensity).

MS+40 mg/L $ADSO_4$		Explants	Colour	Structure	Type	Mean dry weight (g)
NAA	BAP					
0	0	Root	DG	C	E	0.28±0.03b
		Stem	DG	C	NE	0.11±0.01h
		Flower	DG	C	NE	0.12±0.03e
1.0		Root	DG	C	NE	0.12±0.01i
		Stem	DG	C	NE	0.13±0.01efg
		Flower	DG	C	NE	0.11±0.02e
2.0		Root	DG	C	E	0.15±0.01g
		Stem	DG	C	NE	0.13±0.01ef
		Flower	DG	C	NE	0.11±0.02e
3.0		Root	DG	C	NE	0.15±0.01g
		Stem	DG	C	NE	0.23±0.01a
		Flower	DG	C	NE	0.12±0.02e
	1.0	Root	DG	C	NE	0.18±0.03e
		Stem	DG	C	NE	0.13±0.02efg
		Flower	DG	C	NE	0.12±0.03e
	2.0	Root	DG	C	NE	0.16±0.02f
		Stem	DG	C	NE	0.12±0.01efg
		Flower	DG	C	NE	0.11±0.02e
	3.0	Root	DG	C	NE	0.22±0.01d
		Stem	DG	C	NE	0.12±0.01efg
		Flower	DG	C	NE	0.12±0.04e
1.0	1.0	Root	DG	C	E	0.12±0.02i
		Stem	DG	C	NE	0.13±0.01efg
		Flower	DG	C	NE	0.11±0.04e
2.0	**1.0**	**Root**	DG	C	E	**0.31±0.02i**
		Stem	DG	C	NE	0.15±0.02d
		Flower	DG	C	NE	0.18±0.04b
3.0	1.0	Root	DG	C	NE	0.16±0.03f
		Stem	DG	C	NE	0.14±0.01e
		Flower	DG	C	NE	0.18±0.02bc
1.0	2.0	Root	DG	C	NE	0.27±0.02c
		Stem	DG	C	E	0.21±0.01b
		Flower	DG	C	NE	0.14±0.02de
2.0	2.0	Root	DG	C	NE	0.32±0.011a
		Stem	DG	C	NE	0.17±0.01c
		Flower	DG	C	NE	0.12±0.03e
3.0	2.0	Root	DG	C	NE	0.13±0.02h
		Stem	DG	C	NE	0.14±0.01e
		Flower	DG	C	NE	0.16±0.01cd
1.0	3.0	Root	DG	C	NE	0.12±0.02i
		Stem	DG	C	E	0.12±0.02fg
		Flower	DG	C	NE	0.11±0.02e

'**Table 4.1**, continued'

2.0	3.0	Root	DG	C	NE	0.13±0.02h
		Stem	DG	C	E	0.22±0.03ab
		Flower	DG	C	NE	0.14±0.03de
3.0	3.0	Root	DG	C	NE	0.12±0.00i
		Stem	DG	C	NE	0.12±0.01gh
		Flower	DG	C	NE	0.21±0.05a

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). The bold value is represented the best result for each explant (root, stem and flower bud). LG=light green; E=embryogenic; NE=non-embryogenic; C=compact; F=friable.

Table 4.2 : The effects of different concentrations and combinations of 2,4-D and KIN on callus induction from root, stem and flower bud explants of *Clitoria ternatea* L. cultured on MS media added with 40.0 mg/L $ADSO_4$ for 8 weeks. The cultures were maintained at 24±1ºC with 16 hours light and 8 hours dark (1000 lux intensity).

MS+40 mg/L $ADSO_4$		Explants	Colour	Structure	Type	Mean dry weight (g)
2,4-D	KIN					
0	0	Root	DG	C	E	0.14±0.02d
		Stem	DG	C	NE	0.10±0.01i
		Flower	LG	F	NE	0
1.0		Root	DG	C	NE	0.12±0.01fg
		Stem	DG	C	NE	0.12±0.01fg
		Flower	LG	F	NE	0
2.0		Root	DG	C	E	0.11±0.02i
		Stem	DG	C	NE	0.13±0.02d
		Flower	LG	F	NE	0.12±0.03c
3.0		Root	DG	C	NE	0.11±0.02h
		Stem	DG	C	NE	0.13±0.01ef
		Flower	LG	F	NE	0.18±0.07a
	1.0	Root	DG	C	NE	0.34±0.02b
		Stem	DG	C	NE	0.18±0.01a
		Flower	LG	F	NE	0.16±0.03b
	2.0	**Root**	DG	C	NE	**0.43±0.02a**
		Stem	DG	C	NE	0.16±0.01b
		Flower	LG	F	NE	0.17±0.02a
	3.0	Root	DG	C	NE	0.15±0.03c
		Stem	DG	C	NE	0.12±0.01ef
		Flower	LG	F	NE	0.15±0.04b
1.0	1.0	Root	DG	C	E	0.13±0.01e
		Stem	DG	C	NE	0.13±0.01e
		Flower	LG	F	NE	0.11±0.04d
2.0	**1.0**	**Root**	DG	C	E	**0.12±0.01gh**
		Stem	DG	C	NE	0.13±0.01ef
		Flower	LG	F	NE	0.15±0.03b
3.0	1.0	Root	DG	C	NE	0.12±0.01f
		Stem	DG	C	NE	0.12±0.01efg
		Flower	LG	F	NE	0.11±0.01d
1.0	2.0	Root	DG	C	NE	0.11±0.01i
		Stem	DG	C	E	0.15±0.01c
		Flower	LG	F	NE	0.10±0.02d
2.0	2.0	Root	DG	C	NE	0.11±0.02h
		Stem	DG	C	NE	0.12±0.02h
		Flower	LG	F	NE	0.11±0.02d
3.0	2.0	Root	DG	C	NE	0.12±0.01f
		Stem	DG	C	NE	0.12±0.01gh
		Flower	LG	F	NE	0.16±0.03b
1.0	3.0	Root	DG	C	NE	0.12±0.01gh
		Stem	DG	C	E	0.13±0.01ef
		Flower	LG	F	NE	0.12±0.02bc

'**Table 4.2**, continued'

2.0	3.0	Root	DG	C	NE	0.12±0.02fg
		Stem	DG	C	E	0.12±0.01ef
		Flower	LG	F	NE	0.12±0.04c
3.0	3.0	Root	DG	C	NE	0.11±0.01gh
		Stem	DG	C	NE	0.13±0.01ef
		Flower	LG	F	NE	0.13±0.03c

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). The bold value is represented the best result for each explant (root, stem and flower bud). LG=light green; E=embryogenic; NE=non-embryogenic; C=compact; F=friable.

4.3.3 Effects of Various Hormones (NAA, BAP, 2,4-D and KIN) on Callus Induction and Somatic Embryos from Root and Stem Explants of *Onobrychis viciifolia* Scop.

Tables 4.3 and 4.4 showed the responses of root and stem explants of *O. viciifolia* Scop.. Unfortunately, no flowers were produced from the seeds germination of this species. Both root and stem explants formed light green friable callus, under all treatments on $ADSO_4$-added MS media supplemented with NAA, BAP, 2,4-D and KIN (1.0-3.0 mg/L), except on MS+40.0 mg/L $ADSO_4$+1.0 mg/L 2,4-D+3.0 mg/L KIN, white friable callus formation was obtained. The highest callus formation (1.44±0.02 g of non-embryogenic callus) from root explant was observed on MS+40.0 mg/L $ADSO_4$+ 1.0 mg/L NAA+3.0 mg/L BAP, as compared to 1.05±0.01 g embryogenic callus on MS+40.0 mg/L $ADSO_4$+1.0 mg/L NAA+2.0 mg/L BAP. The highest callus formation from stem explant (0.73±0.02 g of embryogenic callus) on MS+40.0 mg/L $ADSO_4$+2.0 mg/L KIN. Overall, more somatic embryos were obtained with additional of 2.4-D and KIN as compared to addition of NAA and BAP. The comparison of embryogenic callus induction of *C. ternatea* L. and *O. viciifolia* Scop. from root and stem explants were showed in Figure 4.2 and Figure 4.3, respectively.

Table 4.3 : The effects of different concentrations and combinations of NAA and BAP on callus induction from root and stem explants of *Onobrychis viciifolia* Scop. cultured on MS media added with 40.0 mg/L $ADSO_4$ for 8 weeks. The cultures were maintained at 24±1ºC with 16 hours light and 8 hours dark (1000 lux intensity).

MS+40 mg/L $ADSO_4$		Explants	Colour	Structure	Type	Mean dry weight (g)
NAA	BAP					
0	0	Root	LG	F	E	0.97±0.02e
		Stem	LG	F	NE	0.13±0.01n
1.0		Root	LG	F	E	0.16±0.01i
		Stem	LG	F	NE	0.17±0.01k
2.0		Root	LG	F	NE	0.18±0.01i
		Stem	LG	F	NE	0.19±0.01i
3.0		Root	LG	F	NE	0.22±0.01h
		Stem	LG	F	NE	0.19±0.01j
	1.0	Root	LG	F	E	0.26±0.01g
		Stem	LG	F	NE	0.13±0.02n
	2.0	Root	LG	F	E	0.30±0.01f
		Stem	LG	F	NE	0.82±0.02b
	3.0	Root	LG	F	NE	0.25±0.01g
		Stem	LG	F	NE	0.65±0.01b
1.0	1.0	Root	LG	F	E	0.24±0.03gh
		Stem	LG	F	NE	0.25±0.02h
2.0	1.0	Root	LG	F	E	1.09±0.01b
		Stem	LG	F	NE	0.58±0.01c
3.0	1.0	Root	LG	F	E	1.05±0.01c
		Stem	LG	F	NE	0.38±0.02f
1.0	2.0	Root	LG	F	E	1.05±0.01c
		Stem	LG	F	NE	0.38±0.02f
2.0	2.0	Root	LG	F	NE	0.97±0.01e
		Stem	LG	F	E	0.13±0.01e
3.0	2.0	Root	LG	F	NE	0.24±0.02g
		Stem	LG	F	NE	0.42±0.02e
1.0	**3.0**	**Root**	LG	F	NE	**1.44±0.02a**
		Stem	LG	F	NE	0.45±0.03d
2.0	3.0	Root	LG	F	NE	1.03±0.02d
		Stem	LG	F	NE	0.15±0.01l
3.0	3.0	Root	LG	F	NE	0.24±0.02g
		Stem	LG	F	NE	0.13±0.02mn

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). The bold value is represented the best result for each explant (root and stem). LG=light green; E=embryogenic; NE=non-embryogenic; C=compact; F=friable.

Table 4.4 : The effects of different concentrations and combinations of N2,4-D and KIN on callus induction from root and stem explants of *Onobrychis viciifolia* Scop. cultured on MS media added with 40.0 mg/L $ADSO_4$ for 8 weeks. The cultures were maintained at 24±1ºC with 16 hours light and 8 hours dark (1000 lux intensity).

MS+40 mg/L $ADSO_4$		Explants	Colour	Structure	Type	Mean dry weight (g)
2,4-D	KIN					
0	0	Root	LG	F	E	0.78±0.04c
		Stem	LG	F	NE	0.18±0.01g
1.0		Root	LG	F	E	0.21±0.01j
		Stem	LG	F	NE	0.18±0.01g
2.0		Root	LG	F	NE	0.21±0.02j
		Stem	LG	F	NE	0.22±0.04e
3.0		Root	LG	F	NE	0.21±0.01j
		Stem	LG	F	NE	0.17±0.01h
	1.0	Root	LG	F	NE	0.65±0.02b
		Stem	LG	F	NE	0.18±0.01g
	2.0	Root	LG	F	NE	0.67±0.02b
		Stem	LG	F	E	0.18±0.01g
	3.0	Root	LG	F	NE	0.67±0.02b
		Stem	LG	F	NE	0.73±0.02a
1.0	1.0	Root	LG	F	E	0.27±0.02f
		Stem	LG	F	NE	0.23±0.01d
2.0	1.0	Root	LG	F	NE	0.26±0.04g
		Stem	LG	F	NE	0.22±0.02e
3.0	1.0	Root	LG	F	NE	0.22±0.03e
		Stem	LG	F	NE	0.43±0.01c
1.0	2.0	Root	LG	F	NE	0.24±0.03h
		Stem	LG	F	E	0.52±0.02b
2.0	2.0	Root	LG	F	NE	0.03±0.01h
		Stem	LG	F	NE	0.23±0.03hi
3.0	2.0	Root	LG	F	NE	0.23±0..1hi
		Stem	LG	F	NE	0.18±0.01h
1.0	**3.0**	**Root**	W	F	E	**0.75±0.04a**
		Stem	LG	F	NE	0.20±0.01f
2.0	3.0	Root	LG	F	NE	0.63±0.03e
		Stem	LG	F	NE	0.21±0.02f
3.0	3.0	Root	LG	F	NE	0.23±0.03hi
		Stem	LG	F	NE	0.21±0.01f

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). The bold value is represented the best result for each explant (root and stem). LG=light green; E=embryogenic; NE=non-embryogenic; C=compact; F=friable.

a) *Clitoria ternatea* L. b) *Onobrychis viciifolia* Scop.

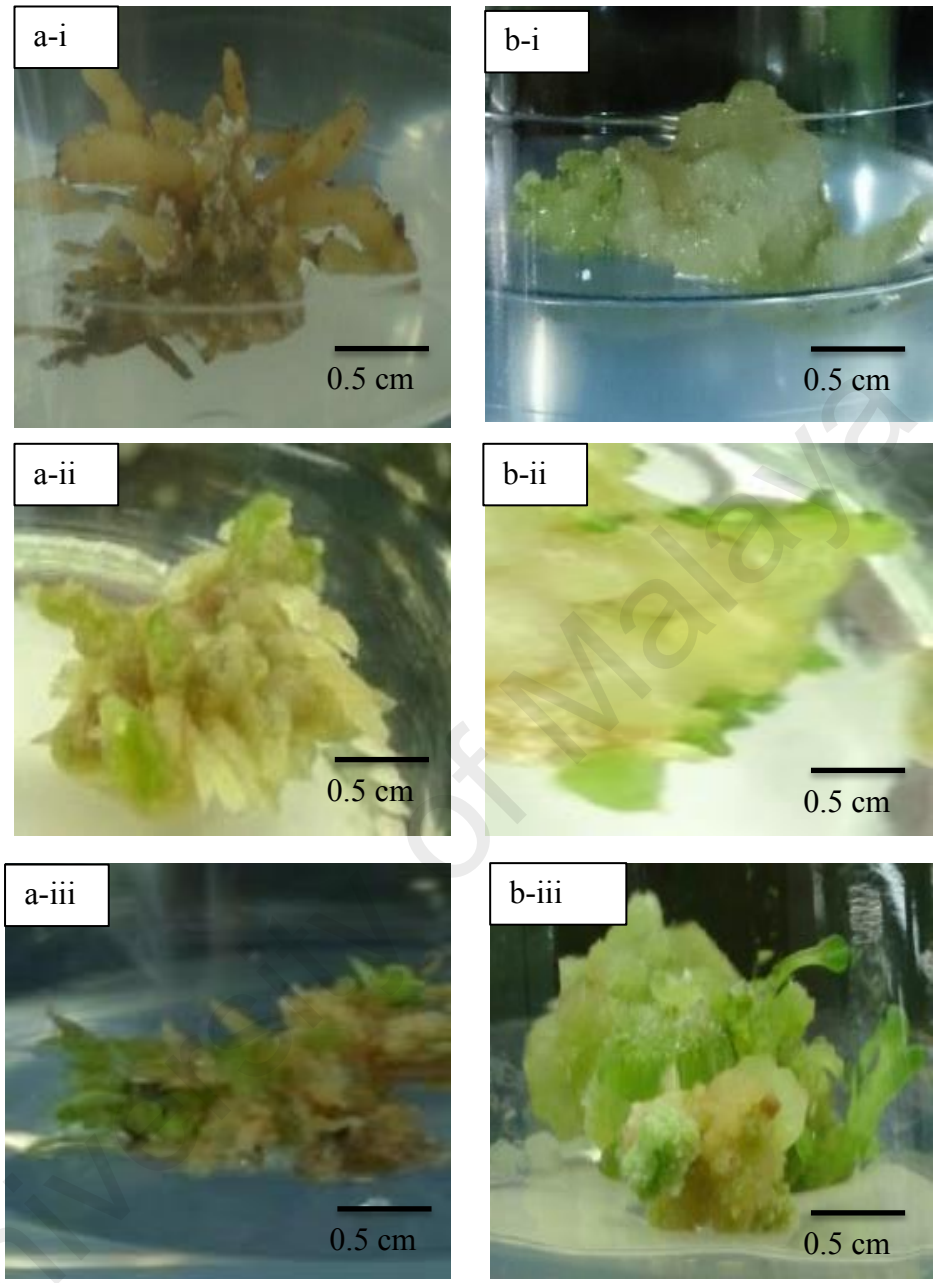


Figure 4.2 : The comparison of somatic embryos initiation and maturation from root explant. a) *Clitoria ternatea* L. cultured on MS+40.0 mg/L $ADSO_4$+2.0 mg/L NAA+1.0 mg/L BAP. b) *Onobrychis viciifolia* Scop. cultured on MS+40.0 mg/L $ADSO_4$+1.0 mg/L NAA+2.0 mg/L BAP ; i) 2-week-old explant. ii) Somatic embryos at different stages of callus clumps (week 4). iii) Multiple shoots formation (week 11).

a) *Clitoria ternatea* L. b) *Onobrychis* viciifolia Scop.

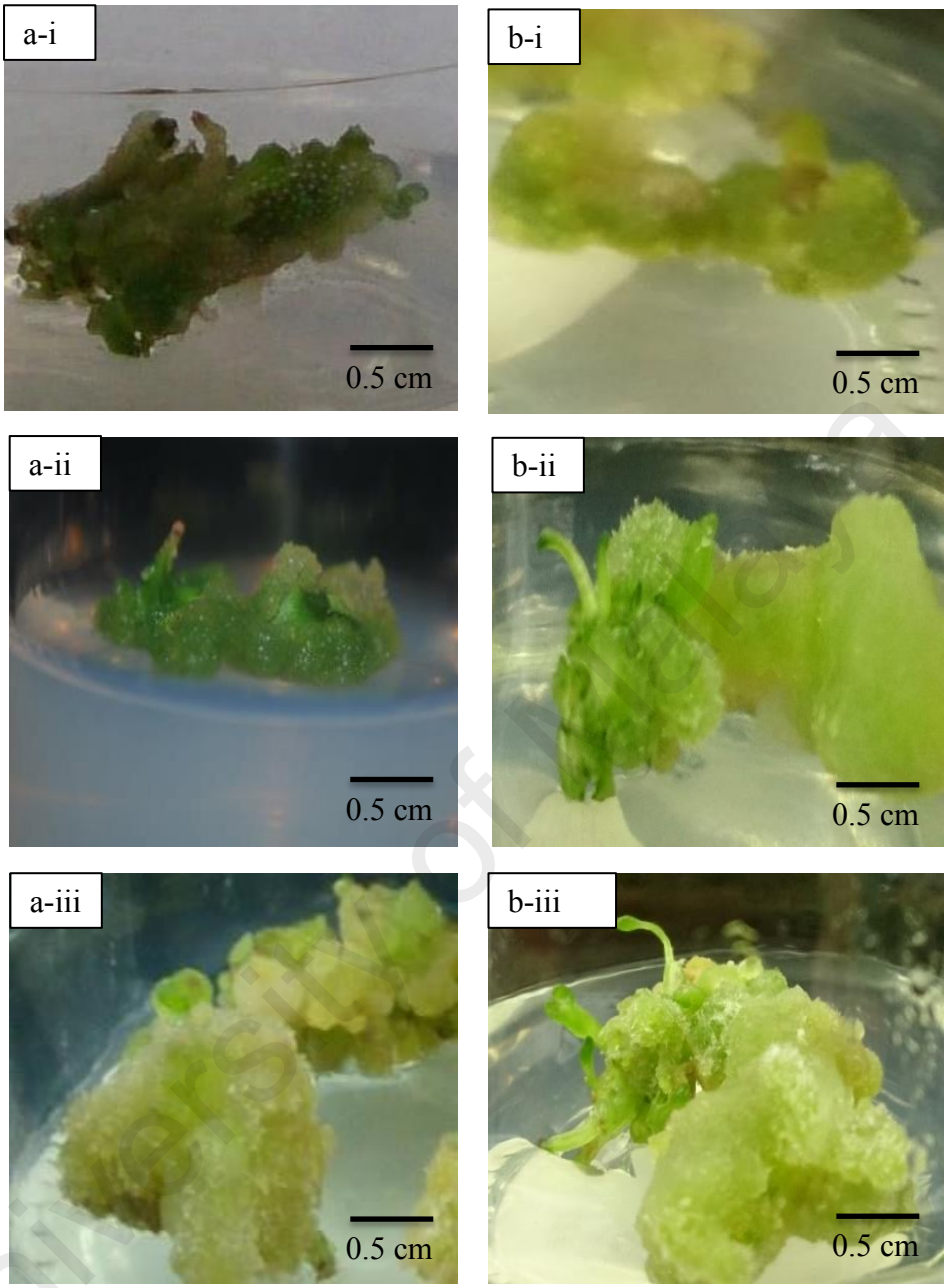


Figure 4.3 : The comparison of somatic embryos initiation and maturation from stem Explant. a) *Clitoria ternatea* L. cultured on MS+40 mg/L $ADSO_4$+3.0 mg/L NAA. b) *Onobrychis viciifolia* Scop. cultured on MS+40.0 mg/L $ADSO_4$+2.0 mg/L KIN.; i) 2-week-old stem explant. ii) Somatic embryogenic at different stage of callus clumps (week 4). Iii) Multiple shoots formation (week 11).

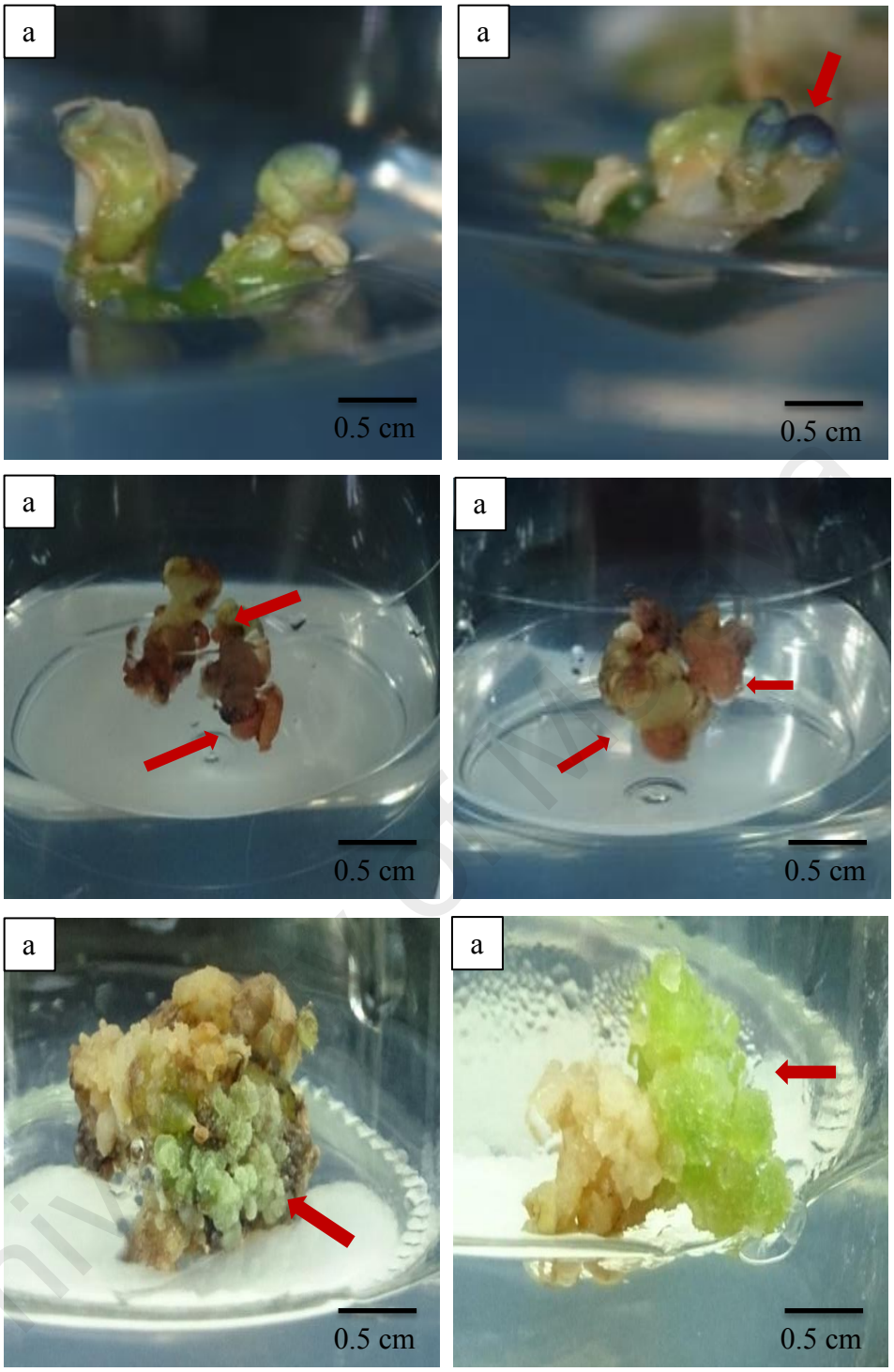


Figure 4.4 : Non-embryogenic callus formation of *Clitoria ternatea* L. from flower bud. (a) Flower bud explant (b) Flower bud explant with vivid blue tip. (c) Explantchange to pink. (d) Growing of pink explant. (e) Dark green coloured of compact callus formation on MS+40.0 mg/L $ADSO_4$+3.0 mg/LNAA+3.0 mg/L BAP. (f) Light green coloured of friable callus on MS+40.0 mg/L $ADSO_4$+3.0 mg/L 2,4-D.

4.3.4 Comparison Between Somatic Embryos of *Clitoria ternatea* L. and *Onobrychis viciifolia* Scop.

Most of the somatic embryos were obtained after 5 weeks being cultured on $ADSO_4$-added MS media. Initially, embryogenic tissue from root, stem and flower bud explants was distinguished by double staining method.

The maturation of somatic embryos via four main stages (globular, heart, torpedo and cotyledonary) of *C. ternatea* L. (Figure 4.5) and *O. viciifolia* Scop. (Figure 4.6) were observed. Generally, *C. ternatea* L took longer time (7-9 weeks) to complete the maturation of somatic embryos compared to *O. viciifolia* Scop. (4-6 weeks). The callus texture of *C. ternatea* L. was harder or more compact when compared with callus of *O. viciifolia* Scop.

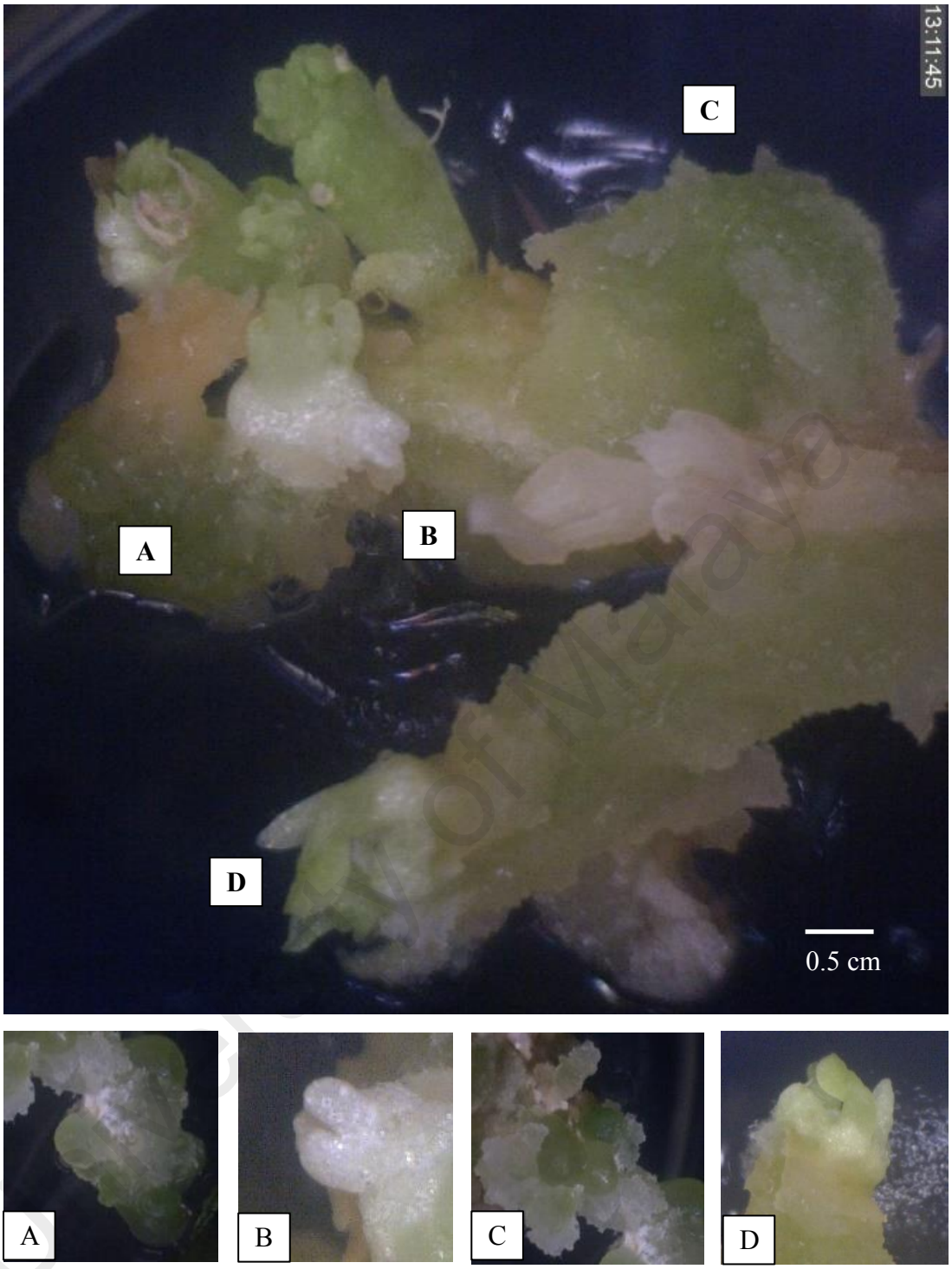


Figure 4.5 : Somatic embryos of *Clitoria ternatea* L. cultured on MS+40.0 mg/L $ADSO_4$+2.0 mg/L NAA+1.0 mg/L BAP, after 8 weeks. A) Globular stage. B) Heart-shape stage. C) Torpedo stage. D) Cotyledonary stage.

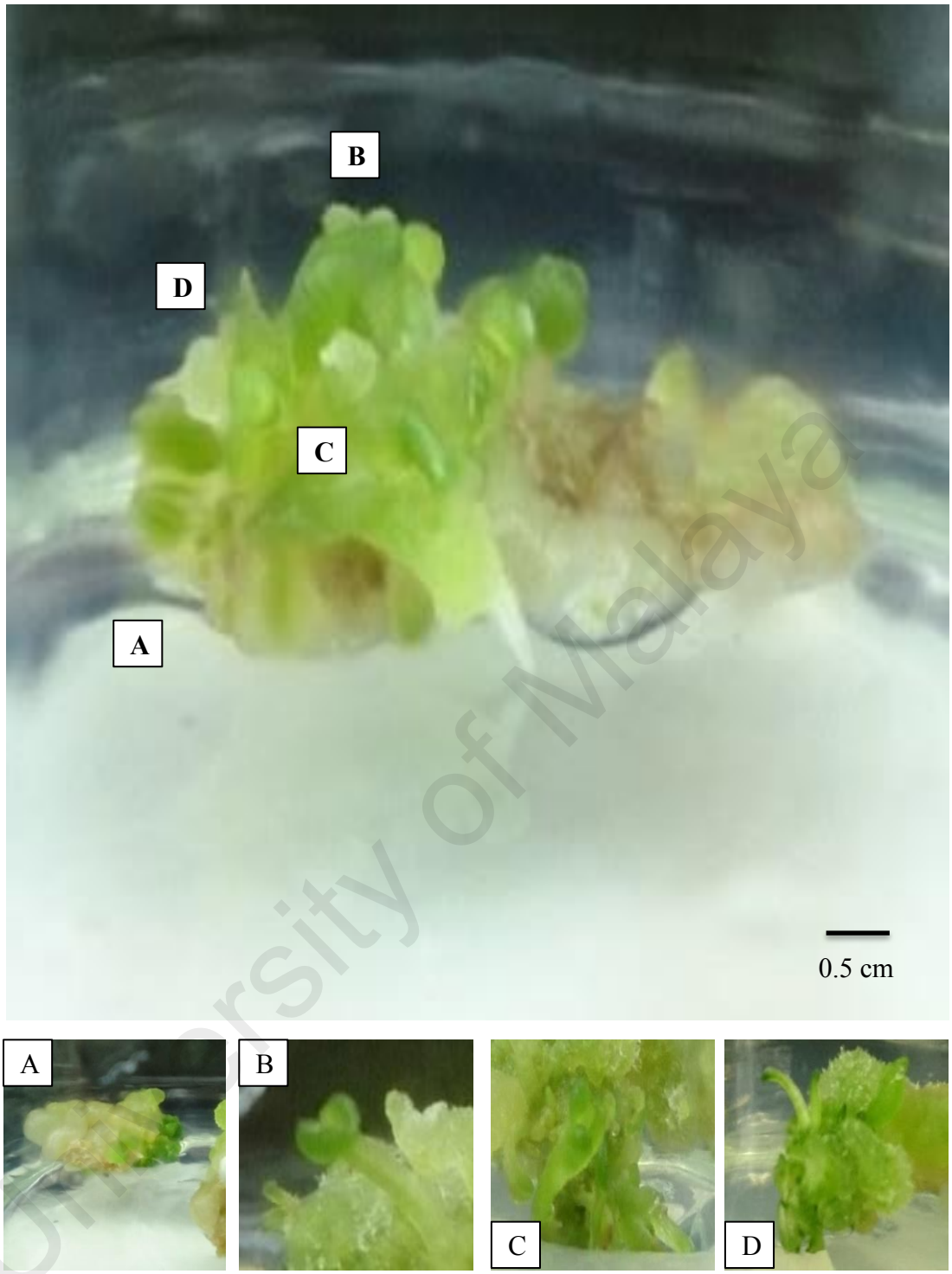


Figure 4.6 : Somatic embryos of *Onobrychis viciifolia* Scop. cultured on MS+40.0 Mg/L ADSO4+1.0 mg/L NAA+2.0 mg/L BAP, after 8 weeks. A) Globular stage. B) Heart-shape stage. C) Torpedo stage. D) Cotyledonary stage.

4.4 SUMMARY OF RESULTS

1. Embryogenic and non-embryogenic callus were determined by double staining method which influenced by age of callus, as well as type and concentrations of hormones. Embryogenic callus formed through four main different stages (globular, heart, torpedo and cotyledonary).
2. The highest weight of non-embryogenic dried callus of *C. ternatea* L. was obtained from root explant (0.43±0.02 g cultured on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$ and 2.0 mg/L KIN), followed by stem explant (0.23±0.01 g cultured on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$ and 3.0 mg/L KIN) and flower bud explant (0.21±0.05 g cultured on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$, 3.0 mg/L NAA and 3.0 mg/L BAP).
3. The highest weight of embryogenic dried callus from root explant of *C. ternatea* L. was obtained on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$, 2.0 mg/L NAA and 1.0 mg/L BAP (0.31±0.02 g), which was lower as compared to *O. viciifolia* Scop. (1.05±0.01 g) cultured on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$, 1.0 mg/L NAA and 2.0 mg/L BAP. (This optimum result was used for synthetic seeds production from somatic embryos in CHAPTER 5).
4. The highest dried weight (0.18±0.07g). of non-embryogenic dried callus (light green) from flower bud explant of *C. ternatea* L. was obtained on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$ and 3.0 mg/L 2,4-D. Generally, the response on colour and texture of callus formation was based on the type of hormones, which formed dark green and compact non-embryogenic callus on MS supplemented with NAA and BAP, but formed light green and friable non-embryogenic callus on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$, 2,4-D and KIN.

5. The highest weight of dried callus of *O. viciifolia* Scop. was obtained from root explant (1.44±0.02 g of non-embryogenic callus cultured on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$, 1.0 mg/L NAA and 3.0 mg/L BAP), followed by stem (0.73±0.02 g of embryogenic callus cultured on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$ and 2.0 mg/L KIN).
6. Overall, root and stem explants of *C. ternatea* L. produced compact dark green callus, while, *O. viciifolia* Scop. formed friable light green callus.
7. *O. viciifolia* Scop. managed to produce three times greater amount of dried callus compared to *C. ternatea* L. within 8 weeks.

CHAPTER 5

SYNTHETIC SEEDS OF *Clitoria ternatea* L. AND *Onobrychis viciifolia* Scop.

5.1 EXPERIMENTAL AIMS

In recent years, somatic embryos are being used for developing synthetic seeds. The development of synthetic seed or encapsulated technology brings up a new prospect in agriculture and nursery industry. Production of synthetic seeds is effective and acts as an important alternative method of propagation in commercially important and endangered plant species. Plants can be produced in large scale with high volumes. Consequently, genetic uniformity and stability of the plant can be maintained. Due to the sterility, it can be easily transported and has potential for long term storage without losing its viability.

The concept of using somatic embryos in synthetic seeds was proposed by Murashige (1978). In 1984, Redenbaugh was reported on encapsulation of somatic somatic embryos in a protective coating with alginate, whereby alfafa (Leguminosae) somatic embryos were mixed with sodium alginate and dropped into a calcium salt solution to form calcium alginate capsules. According to Redenbaugh (1993), alginate, the most widely used hydrogel for encapsulation of embryo, this dissolves easily and remains stable at room temperature.

Although, naturally *C ternatea* L. and *O. viciifolia* Scop. produced a lot of seeds, however, conventional propagation is highly unreliable due to poor seeds germination and failure of young seedlings to survive under natural conditions (Rout, 2005). Synthetic seed technology (for regeneration and conservation) consists of somatic embryos surrounded by an artificial layer called capsule. These somatic embryos, as with zygotic embryos, are possible to be stored for a period of time through synthetic seed technology. Synthetic seeds are beneficial for a long period of storage under low temperatures (4±1ºC) and for mass propagation and

conservation (Datta and Potrykus, 1989; Li et al, 1990, Arrilaga et, al., 1994; Taha et al., 2013). Cold exposure for up to 12 weeks did not affect conversion rate of alfafa embryos in soil under controlled conditions (Fujii et al., 1993). The advantages of synthetic seeds including seeding of clonal varieties and maintenance of elite germplasms, besides for easy handling and transportation, higher scale up capacity and uniformity in production.

The main objectives of this specific chapter were:

1. To determine the optimum concentrations of sodium alginate ($NaC_6H_7O_6$) and soaking period in calcium chloride dehydrate ($CaCl_2.2H_2O$) on bead formation from embryogenic callus of *C. ternatea* L. and *O. viciifolia* Scop.
2. To investigate the effects of low temperature storage (4±1ºC) on synthetic seeds of *C. ternatea* L. and *O. viciifolia* Scop.
3. To compare germination process and morphological characteristics of *C. ternatea* L. and *O. viciifolia* Scop. cultured on solid, liquid and double-layered MS media.

5.2 MATERIALS AND METHODS

5.2.1 Plant Materials and Culture Conditions

Based on the results in Chapter 4, the optimum embryogenic callus (6-week-old) obtained from root explants of *C. ternatea* L. (produced 0.31±0.02 g embryogenic callus cultured on MS+40 mg/L $ADSO_4$+2.0 mg/L NAA+1.0 mg/L BAP) and *O. viciifolia* Scop. (produced 1.05±0.01 g embryogenic callus cultured on MS+40.0 mg/L $ADSO_4$+1.0 mg/L NAA+2.0 mg/L BAP) was selected for creation of synthetic seeds. The cultures were incubated and maintained at 24±1°C with 16 hours light and 8 hours dark. Thirty replicates were used for each treatment. Illumination was at 1000 lux and relative humidity was 90–100%.

5.2.2 Preparation of Culture Media

5.2.2.1 Preparation of MS Stock Media

MS stock media for synthetic seeds production was adapted from Murashige and Skoog (1962). MS stock media consists of macronutrients without calcium, micronutrients, vitamins and iron were prepared in different conical flasks.

Table 5.1: Formulations of MS stock media for synthetic seeds production.

Nutrient	Concentration (g/L)
A. Macronutrients without calcium	
-Ammonium nitrate, NH_4NO_3	16.5
-Potassium nitrate, KNO_3	19.0
-Magnesium sulphate, $MgSO_4.7H_2O$	3.7
-Potassium dihydrogen orthophosphate, KH_2PO_4	1.7
B. Micronutrients	
-Manganese sulphate, $MnSO_4.4H_2O$	1.56
-Zinc sulphate, $ZnSO_4.7H_2O$	0.86
-Potassium iodide, KI	0.083
-Cupric sulphate, $CuSO_4.5H_2O$	0.0025
-Sodium molybdate, $Na_2MoO_4.2H_2O$	0.025
-Cobaltus chloride, $CoCl_2.2H_2O$	0.0025
-Boric acid, H_3BO_3	0.062
C. Vitamins	
-Nicotinic acid	0.05
-Thiamine HCl	0.5
-Pyridoxine HCl	0.5
D. Irons	
-Sodium EDTA Ferric salt, Na_2EDTA	3.73
-Ferrous sulphate, $FeSO_4.7H_2O$	2.78

5.2.2.2 Preparation of Sodium alginate Solution ($NaC_6H_7O_6$)

The protocol for synthetic seeds production was described by Taha et al. (2013) using 1%, 2%, 3%, 4% and 5% (w/v) of the concentrations. Sodium alginate ($NaC_6H_7O_6$) solution as an encapsulation matrix was prepared according to the heating method by Fabre and Dereuddre (1990). To prepare 1% (w/v) $NaC_6H_7O_6$ solution in 100 ml MS basal medium without calcium chloride dehydrate ($CaCl_2.2H_2O$), 1.0 g $NaC_6H_7O_6$ powder was dissolved gradually. Sucrose (3.0 g) and hormones ($ADSO_4$, NAA and BAP) were added. Media pH was adjusted to 5.8. This solution was autoclaved for 20 minutes at 121°C and 104kpa.

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

Calcium chloride dehydrate ($CaCl_2.2H_2O$) solution was used as a complexion agent. To prepare 100 mM (w/v) $CaCl_2.2H_2O$ in 1 L distilled water, 14.7 g $CaCl_2.2H_2O$ powder was dissolved gradually. This solution was autoclaved for 21 minutes at 121°C and 104kpa. Cold solution was used to soak in the beaded alginate in different time period.

5.2.2.4 Encapsulation Technique of Embryogenic Callus

Encapsulation matrix method by Lynch et al. (1996) was using autoclavable micropipette as a tool for formation of beads with 5.0 mm of pipette tip. First, micro shoots sank in $NaC_6H_7O_6$ solution. Then, 3 ml of $NaC_6H_7O_6$ solution with one micro shoot was suck in the syringe before dropped in to $CaCl_2.2H_2O$ solution. Clear beads automatically formed and floating in the calcium chloride. Within 30 minutes, blur beads will sink and were rinsed 3 times with sterile distilled water to wash out the excess $CaCl_2.2H_2O$ solution. Beads were sieved using sterile nylon mesh and blotted with sterile tissue paper.

5.2.2.5 Low Temperature Storage and Survival Rate

Sterile synthetic seeds (beads) were stored in the fridge at 4±1°C (low temperature) and covered with aluminium foil (dark condition) on solid and liquid MS media. The survival rates for germination after 15, 30, 45, 60, 75 and 90 days on solid, liquid and double-layered MS media, at 24±1°C under 16 hours light, were recorded. Thirty replicates were used in each treatment.

5.2.3 Data Analysis

All experiments were conducted using a completely randomized design. Data collected were statistical analyzed using Duncan's Multiple Range Test (DMRT). Mean with different letters in the same column differ significantly at p=0.05.

5.3 RESULTS

5.3.1 Identification of Suitable Concentration of Sodium Alginate Encapsulation Matrix

As the results obtained in Table 5.2, the current study shows that different concentrations of sodium alginate ($NaC_6H_7O_6$) and soaking duration of calcium chloride dehydrate ($CaCl_2.2H_2O$) influenced the bead formation of *C. ternatea L and O. viciifolia Scop.*. Under sterile condition, embryogenic callus were encapsulated using sodium alginate solution for bead formation with ideal texture with uniform, isodiametric shape and size. The optimum concentration for the formation of encapsulation matrix was 3.0% (w/v) $NaC_6H_7O_6$. Encapsulated callus were soaked in calcium 100 mM (w/v) $CaCl_2.2H_2O$ solution for 30 minutes. No suitable beads were formed with low concentration (1-2 %) of sodium alginate. No definite shape of beads was formed within 10 minutes soaking in calcium chloride dehydrate. After 20 minutes in calcium chloride dehydrate, beads were clear, solid and with round shape at the outside, however, the inside bead was still with liquid condition which caused problems when the beads were cultured on solid MS basal media, the beads became shrunk and explants died. The optimum soaking period was 30 minutes in calcium chloride dehydrate with high concentration of 3% (w/v) of sodium alginate which formed very hard beads but perfect round shape. The beads of *Clitoria ternatea* L. (containing 3% w/v of sodium alginate supplemented with 40.0 mg/L ADSO4+2.0 mg/L NAA+1.0 mg/L BAP) and *Onobrychis viciifolia* Scop. (containing 3% (w/v) of sodium alginate supplemented with 40.0 mg/L ADSO4+1.0 mg/L NAA+2.0 mg/L BAP) were cultured on the solid MS media supplemented with the same hormone in the beads for shoots and roots formation, as the basic medium. After 20 weeks, the regenerants from synthetic seeds were transferred to the green house for acclimatization on black garden soil.

Table 5.2 : Effects of different concentrations of sodium alginate ($NaC_6H_7O_6$) and soaking time in calcium chloride dehydrate ($CaCl_2.2H_2O$) on bead formation.

Soaking period in $CaCl_2.2H_2O$ (minutes)	Concentration of $NaC_6H_7O_6$ (%)				
	1.0	2.0	3.0	4.0	5.0
10	-	+	++	++	++
20	-	+	++	++	++++
30	-	++	+++	++++	*
40	-	++	++++	++++	*

- No bead
+ Very fragile bead, no definite shape
++ Soft and oval bead
+++ Solid and round bead
++++ Hard and round bead
• Too hard and round bead

5.3.2 Identification of Effect of Low Temperature Storage (4±1ºC) on Synthetic Seeds Germination

Table 5.3 showed the effect of storage periods on germination and survival rate of embryogenic callus of *C. ternatea* L. *and O. viciifolia* Scop. in 3% (w/v) sodium alginate encapsulated in 90 days, at low temperature storage (4±1ºC). Overall, synthetic seeds of *C. ternatea* L. germinated better (24.9±0.8 to 30.0±0.0 number of seeds) as compared to *O. viciifolia* Scop. (19.7±2.3 to 30.0±0.0 number of seeds), in 90 days. However, the viability of synthetic seeds of *C. ternatea* L. was reduced after 45 days to 28.2±0.6 number of seeds, but still with 100% survival rate, whilst the germination of *O. viciifolia* Scop. was reduced for every 15 days interval. The results showed 100% survival rate of *C. ternatea* L. for 45 days as compared to *O. viciifolia* Scop. (100% germination rate only for 30 days). The minimum survival rate for *C. ternatea* L. (61.7±0.9) was three times higher than *O. viciifolia Scop*.(19.2±0.3). In addition, the germination rate of *C. ternatea* L. (24.9±0.8) showed two times higher than *O. viciifolia* Scop. (19.7±2.3). Therefore, the storage of synthetic seed at 4±1ºC was more preferable to *C. ternatea* L. compared

to *O. viciifolia* Scop., which supported the criteria from encapsulated embryogenic callus of *C. ternatea* L. to viable and survive under low temperature condition, though the original habitat was at high temperature (temperate regions).

Table 5.3 : Effect of storage duration on germination and survival rate of embryogenic callus of *Clitoria ternatea* L. *and Onobrychis viciifolia* Scop. in 3% (w/v) sodium alginate encapsulated for 90 days.

Period of storage (day)	No. of germination	Survival rate (%)	No. of germination	Survival rate (%)
0	30.00±0.00a	100.00±0.00a	30.00±0.00a	100.00±0.00a
15	30.00±0.00a	100.00±0.00a	29.20±0.60b	100.00±0.00a
30	30.00±0.00a	100.00±0.00a	25.80±0.50c	100.00±0.00a
45	28.20±0.60b	100.00±0.00a	24.10±0.60d	62.00±0.00b
60	27.30±1.30c	89.50±2.40b	22.90±0.70e	37.00±0.00c
75	26.70±0.20d	78.40±1.30c	21.20±0.40f	29.30±0.10d
90	24.90±0.80e	61.70±0.90d	19.70±2.30g	19.20±0.30e

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). n=30.

5.3.3 Synthetic Seeds Germination, *In Vivo* and *In Vitro*

Results in Table 5.4 showed the synthetic seeds germination from embryogenic callus of *C. ternatea* L. (Figure 5.1) and *O. viciifolia* Scop. (Figure 5.2) grown *in vivo* (sterile black soil, non-sterile black soil, sterile distilled water and non-sterile distilled water) and *in vitro* (solid, liquid and double-layered MS media). Overall, synthetic seeds of *O. viciifolia* Scop. (3-5 days) were germinated faster than *C. ternatea* L. (7-9 days) on MS media (either solid or liquid). The best synthetic seed germination in shortest time was on liquid and double-layered MS media for *O. viciifolia* Scop. and *C. ternatea* L., 3 and 7 days, respectively. The slowest germination (16-17 days) for both species was observed in sterile distilled water. No germination was observed on soil and water (sterile and non-sterile), grown *in vivo*. The synthetic seeds became shrunk and contaminated by bacteria or fungi. Although, abnormal growth was observed on double-layered MS medium, where the plants (leaves and stem) became brittle and fragile (crunchy texture), however, the growth was super vigorous as compared to solid and liquid MS

medium alone. Within 3-5 months, the synthetic seeds were maintained viable. This result suggested that both liquid and double-layered MS media were suitable for synthetic seeds storage condition, besides dark and low temperature (4±1°C). Normally, synthetic seeds were only stored on sterile solid MS medium to avoid shrinkage and contamination.

Table 5.4 : Synthetic seeds germination from embryogenic callus (initiated from root explant) of *Clitoria ternatea* L. and *Onobrychis viciifolia* Scop. grown *in vivo* and *in vitro*.

Sowing substrate	Observations	
	Clitoria ternatea L.	*Onobrychis viciifolia* Scop.
MS solid	Normal growth. Synthetic seeds germinated within 9 days.	Normal growth. Synthetic seeds germinated within 5 days.
MS liquid	Vigorous growth. Synthetic seeds germinated within 7 days.	Vigorous growth. Synthetic seeds germinated within 3 days.
MS double-layered	Vigorous and normal growth. Synthetic seeds germinated within 7 days.	Vigorous and abnormal growth, with crunchy texture. Synthetic seeds germinated within 3 days.
Sterile black soil	No germination. Synthetic seeds became and contaminated.	No germination. Synthetic seeds shrunk and contaminated.
Non-sterile black soil	No germination. Synthetic seeds shrunk and contaminated.	No germination. Synthetic seeds shrunk and contaminated.
Sterile distilled water	Slow germination. Synthetic seeds germinated within 17 days.	Slow germination. Synthetic seeds germinated within 16 days.
Non-sterile distilled water	No germination. Synthetic seeds contaminated.	No germination. Synthetic seeds contaminated.

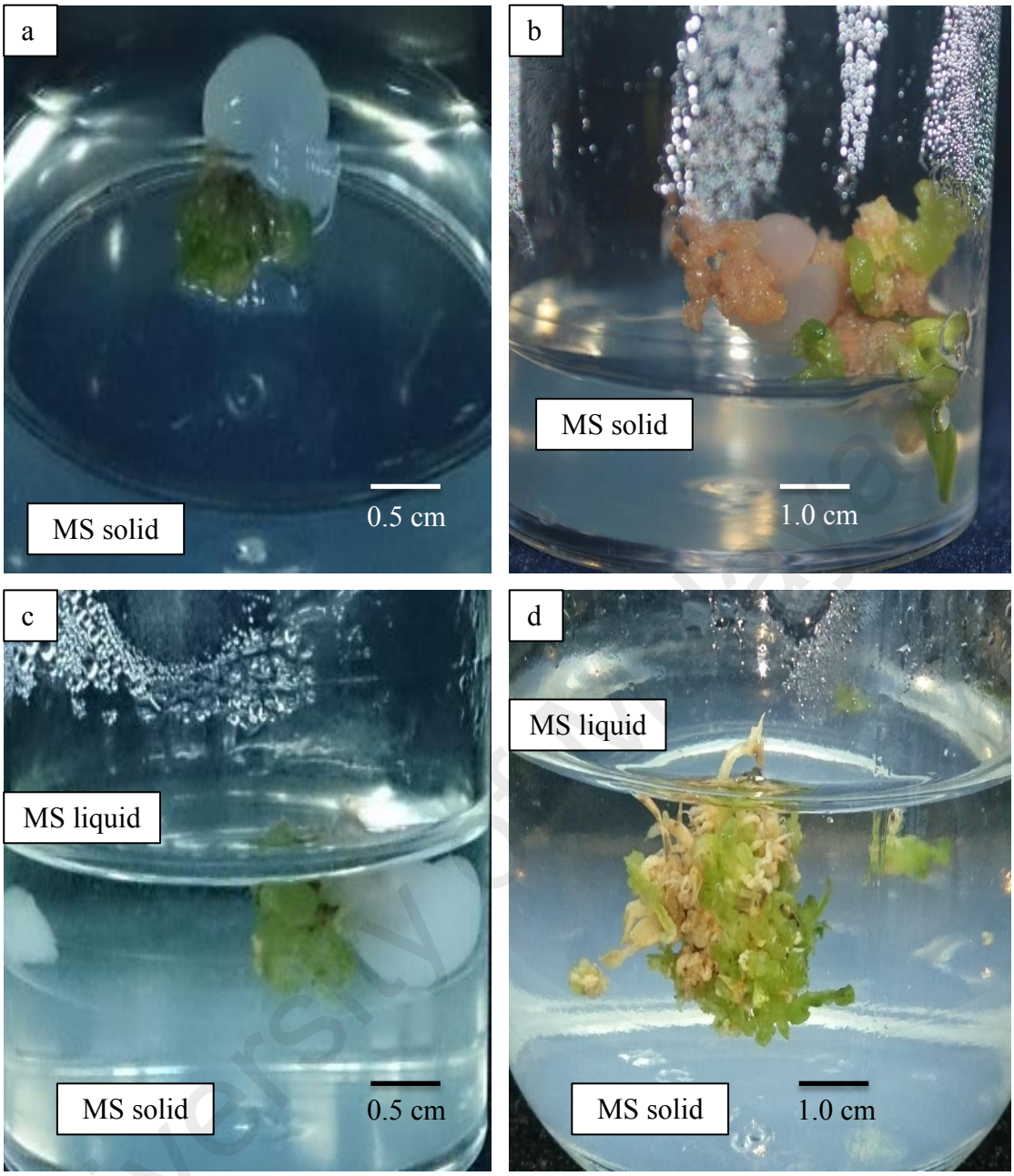


Figure 5.1: Synthetic seeds germination from embryogenic callus of *Clitoria ternatea* L. containing 3% (w/v) sodium alginate with MS+40.0 mg/L $ADSO_4$+2.0 mg/L NAA+1.0 mg/L BAP from low temperature storage (4±1ºC) for 3 months. (a) Synthetic seed on solid MS media. (b) Germination of synthetic seed on solid MS media. (c) Synthetic seed on double-layered MS media. (d) Germination of synthetic seed on double-layered MS media.

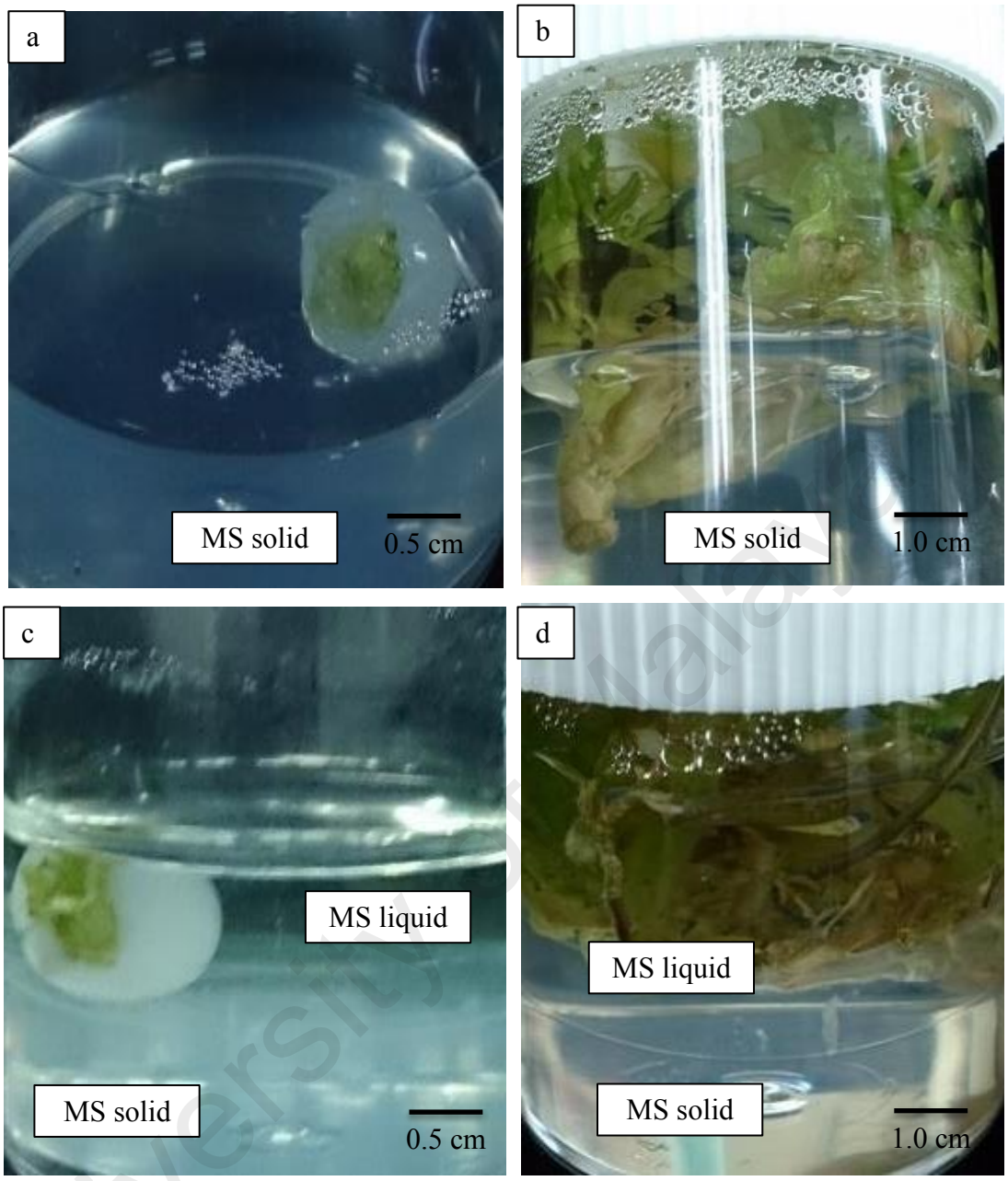


Figure 5.2 : Synthetic seeds germination from embryogenic callus of *Onobrychis viciifolia* Scop. containing 3% (w/v) sodium alginate with MS+40.0 mg/L $ADSO_4$+1.0 mg/L NAA+2.0 mg/L BAP from low temperature storage (4±1ºC) for 3 months. (a) Synthetic seed on solid MS media. (b) Germination of synthetic seed on solid MS media. (c) Synthetic seed on double-layered MS media. (d) Germination of synthetic seed on double-layered MS media.

5.4 SUMMARY OF RESULTS

1. The optimum concentration for the formation of encapsulation matrix was 3.0% (w/v) sodium alginate ($NaC_6H_7O_6$) soaked in 100 mM (w/v) calcium chloride dehydrate ($CaCl_2.2H_2O$) solution for 30 minutes.

2. Synthetic seeds from embryogenic callus (6-week-old) of *C. ternatea* L. germinated better (24.9±0.8 to 30.0±0.0 number of seeds) as compared with *O. viciifolia* Scop. (19.7±2.3 to 30.0±0.0 number of seeds), in 90 days.

3. After three months, the synthetic seeds were still viable for germination of *C. ternatea* L. (61.7±0.9% germination rate) and *O. viciifolia* Scop. (19.2±0.3% germination rate).

4. The viability of synthetic seeds of *C. ternatea* L. was reduced after 45 days of exposure to low temperature storage (4±1ºC) with 28.2±0.6 number of seeds, but still with 100% survival rate, while, the germination of *O. viciifolia* Scop. was reduced for every 15 days interval.

5. Synthetic seeds of *O. viciifolia* Scop. (3-5 days) were germinated faster than *C. ternatea* L. (7-9 days) on MS media (either solid or liquid).

6. Although, abnormal growth was observed on double-layered MS medium, where the plants (leaves and stem) became brittle and fragile (crunchy texture), however, the growth was super vigorous as compared to solid and liquid MS medium alone.

CHAPTER 6

ACCLIMATIZATION AND MACROMORPHOLOGICAL STUDIES OF *Clitoria ternatea* L. AND *Onobrychis viciifolia* Scop.

6.1 EXPERIMENTAL AIMS

Acclimatization of regenerants from *in vitro* to *ex vitro* condition is an adaptation process to the natural environment for various plant species which has undergone growth and development process *in vitro* (Preece and Sutter, 1991). In tissue culture system, most of the acclimatized *in vitro* plantlets showed almost similar characteristics as intact plants (Mohammed and Vidaver, 1990). Plant morphology is the study of the physical form and external structure (macromorphology) and ultrastructure (micromorphology) for taxonomic and identification information. The importance of macromorphology and micromorphological features for the taxonomic consideration of angiosperms is recently mounting up (Parveen et. al., 2000). Micromorphological parameters of different plant parts have been used as aids in the taxonomical recognition of species (Kathiresan et. al., 2011). Macromorphologically, the most interesting characteristic in Leguminosae (including *Clitoria ternatea* L. and *Onobrychis viciifolia* Scop.) is the symbiotic association with N-fixing rhizobia bacteria which live in small growths attached to the plant roots called nodules. Most perennial legumes showed finger-like shaped nodules, which contributing for biological nitrogen fixation (change N_2 to NH_3) and in return, the plant contributes the nutritional and energy (photosynthesis-derived sugar) for the bacteria.

The main objectives of this specific chapter were:

1. To investigate the growth performance of two Leguminosae species (*C. ternatea* L. and *O. viciifolia* Scop.) originated from two different regions (tropical and temperate), grown *in vivo*, *in vitro* and *ex vitro*.

2. To investigate the root systems and nodules formation of *C. ternatea* L. and *O. viciifolia* Scop., grown *in vivo* and *in vitro*.

3. To establish the optimum adaptation through acclimatization process of the

plantlets from *in vitro* system to the natural environment.

4. To compare the growth performances of vegetative (stems, leaves, and roots) and reproductive (flowers and fruit pods) system of *C. ternatea* L. and *O. viciifolia* Scop., grown *in vivo*, *in vitro* and *ex vitro*.

6.2 MATERIALS AND METHODS

6.2.1 Plant Materials of *In Vitro* Regeneration

The acclimatization protocols were established by adapting field transfer method for *Solanum melongena* (Taha and Tijan, 2002), with minor modifications. After seven months in cultures (*in vitro*), all regenerated plantlets (complete with roots and shoots) were transferred to the green house (natural environment) for further growth and development. For the first 2-3 weeks, plantlets were transferred to the soil and covered with plastic in the culture room. After that, plantlets were exposed to the natural environment in the green house. In this experiment, the plantlets obtained from CHAPTER 3 derived from root explants with embryogenic callus cultured on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$, 2.0 mg/L NAA and 1.0 mg/L BAP, as well as on MS medium supplemented with combinations of 40.0 mg/L $ADSO_4$, 1.0 mg/L NAA and 2.0 mg/L BAP for *C. ternatea* L. and *O. viciifolia* Scop, respectively.

6.2.2 Transferring Plantlets to Soils

Plantlets (7-month-old) of *C. ternatea* L. *and O. viciifolia* Scop. derived from tissue culture techniques were transferred onto five types of commercial soils (purchased from Fern Garden Nursery, Sungai Buloh, Selangor) in the containers and

exposed to the natural environment in the green house for acclimatization process. Plantlets were grown in containers and covered with plastic (tightened with rubber band to avoid stress conditions and relatively low humidity environment) in the culture room for 3 weeks before being transferred to the green house.

6.2.2.1 Effects of Soil Types and pH on Survival Rates

In this experiment, 7- month-old plantlets were transferred to eight types of soils (with particular pH) in the green house at 30±1°C (Malaysian climate). Thirty replicates were used and the survival rates were compared.

6.2.3 Effects of Growth Performances

The growth performance based on macromorphological features were observed to compare the characteristics between *in vivo* (mother), *in vitro* (regenerated) and *ex vitro* (acclimatized) plants.

6.2.4 Data Analysis

All experiments were conducted using a completely randomized design. Data collected were statistical analyzed using Duncan's Multiple Range Test (DMRT). Mean with different letters in the same column differ significantly at $p<0.05$.

6.3 RESULTS

6.3.1 Determination of Soil Types and pH on Survival Rates of *Clitoria ternatea* L. and *Onobrychis viciifolia* Scop.

Table 6.1 exhibited the survival rates of *C. ternatea* L. and *O. viciifolia* Scop. on different soil types, after one month being acclimatized (*ex vitro*) under natural environment (30±1ºC). Initialy, the plantlets were maintained for 7 months *in vitro* condition (cultured on MS media supplemented with $ADSO_4$, NAA and BAP). Generally, for all tested soils, the survival rates of plantlets of *C.ternatea* L. (64.71-91.07%) was better than *O. viciifolia* Scop. (39.22-71.58%). The highest survival rate (91.07±0.96%) obtained from plantlets of *C. ternatea* L., transferred on mud soil (pH4.26±0.12), whilst, plantlets of *O. viciifolia* Scop. survived the best on topsoil (71.58±1.27%). The lowest survival rate was on clay loam soil (pH 6.03±0.29) for both *C. ternatea* L. and *O. viciifolia* Scop. with 64.71±1.01% and 39.22±0.97%, respectively.

Table 6.1 : Effects of soil types, pH and survival rates on plantlets of *Clitoria ternatea* L. and *Onobrychis viciifolia* Scop., after one month being acclimatized.

Soil types	Mean pH	Mean survival rate, %	
		Clitoria ternatea L. (tropical legume)	*Onobrychis viciifolia* Scop. (temperate legume)
Mud Soil	4.26±0.12	**91.07±0.96a**	42.03±2.51d
Burn Soil	4.89±0.11	83.42±1.49b	56.41±1.33b
Topsoil	5.14±0.12	79.98±1.50c	**71.58±1.27a**
Clay Loam soil	6.03±0.29	64.71±1.01f	39.22±0.97e
Black Soil	5.08±0.18	75.34±1.38d	51.05±1.52c
Laterite Soil	6.38±0.27	67.21±2.44e	43.82±2.14d

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). n=30.

6.3.2 Acclimatization and Macromorphology of *Clitoria ternatea* L.

The regenerated plantlets were removed from the culture containers carefully. The roots were washed to get rid of the agar. For the first 3 weeks, plantlets of *C. ternatea* L. were acclimatized on mud soil in the culture room at 24±1ºC for

adaptation or acclimatization process. Then, the plantlets were transferred to the green house at 30±1ºC.

Table 6.2 and Figure 6.1 showed the differences in size of stem height and leaf width, number of flowers and number of nodules between *in vivo*, *in vitro* and *ex vitro* grown plants of *C. ternatea* L. At the same age of 7-month-old, *in vivo* plants showed the highest stem (95.03±0.83 cm**)**. Even though, the *ex vitro* plants was shorter (39.16±0.28 cm), which was about half as compared to the *in vivo* plant height, however, the quality of leaves (2.11±0.11 cm) and flowers (9-16 flowers per plant) were the best among the three growth conditions.

Moreover, in this study, only *ex vitro* plants managed to form nodules (25-40 nodules per plant) which started after 6 weeks being transferred to soil consist nodules of white and orange nodules attached to the secondary roots. A week later, the first twiner was formed. After 12 weeks, the plantlets started to flower and fruiting (after 3 months) which were in shorter time as compared to the *in vivo* plants (5-6 months). The *in vivo* primary roots were long and large (8.7-15.2 cm length and 0.2-0.3 cm width) and the young or white nodules (10-35) were observed at the secondary roots. The white nodules change to orange nodules within 6-7 days. No nodule *in vitro* conditions, but node structure. The *ex vitro* primary roots were shorter (7.2-12.5 cm length), but as width as *in vivo* grown plant (0.2-0.3 cm width). Nodules only observed after 2 months being acclimatized, but with small quantity (8-13 nodules). Finally, the plants growth and structures were the same as the mother plant (*in vivo*).

Table 6.2 : The comparison of growth performance and structures of stems, leaves, flowers and nodules of *Clitoria ternatea* L., grown *in vivo*, *in vitro* and *ex vitro*.

Macromorphology	Observations		
	In vivo (7-month-old mother plant)	*In vitro* (7-month-old regenerated plant)	*Ex vitro* (7-month-old acclimatized plant)
A.VEGETATIVE SYSTEM			
Stems			
Description	Herbaceous fine twiner and woody stems (5-7 m).	No twiner and woody stem.	Herbaceous fine twiner and woody stems (3-5 m).
Stem height (cm)	**95.03±0.83a**	12.47±0.62c	39.16±0.28b
Leaves			
Description	Pinnate with 5 dark green leaflets in nearly triangular shape.	Leaflets with darkest green and round in shape.	Pinnate with 5 darker green leaflets in oval shape.
Leaves width (cm)	1.52±0.77ab	0.59±0.33b	**2.11±0.11a**
Root			
Description	Longest and largest-(8.7-15.2 cm length and 0.2-0.3 cm width) primary roots with one deep tap root. Nodules at the secondary roots.	Long (3.5-4.5 cm length and 0.1-0.2 cm width) primary roots with one deep tap root. Nodes at the secondary roots.	Longer (7.2-12.5 cm length and 0.2-0.3 cm) primary roots with one deep tap root. Nodules at the secondary root.
Number of nodules	10-35	0	25-40
B.REPRODUCTIVE SYSTEM			
Flowers			
Description	Single flowerswith dark vivid blue colour and thick petals.	No flower.	Single flowers with darker vivid blue colour and thicker petals.
Number of flowers	5-10	0	**9-16**
Fruit pods			
Description	Dark green flattened pea-like pod with 5 seeds per pod.	No fruit pod.	Darker green flattened pea-like pod with 7 seeds per pod.
Fruit pod length (cm)	3.0-5.0	0	5-10

Mean values with different letters within a row are significantly different at p=0.05, by one way ANOVA and Duncan's Multiple Range Test (DMRT). n=30.

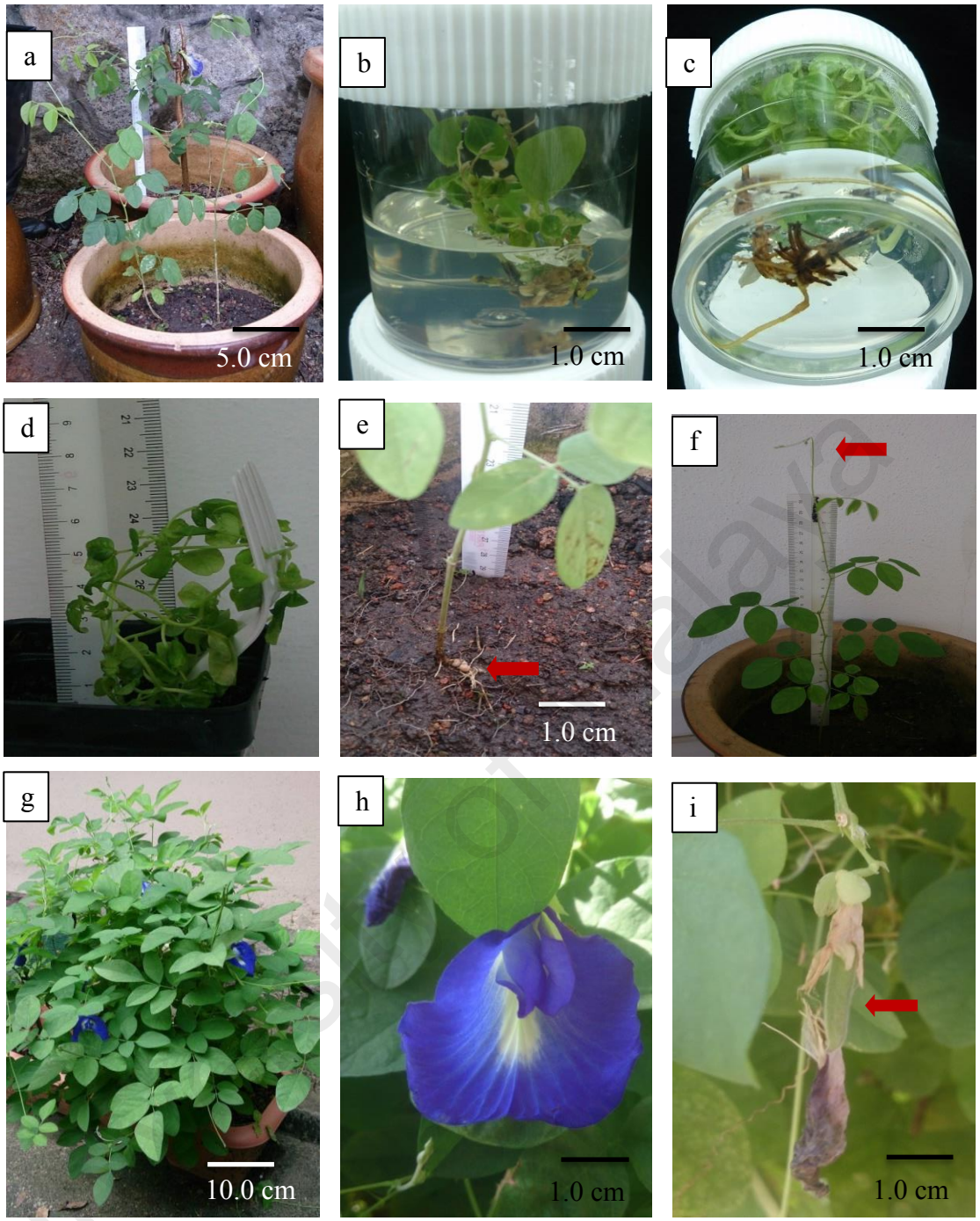


Figure 6.1 : *In vitro* regeneration and acclimatization of *Clitoria ternatea* L. from root explant. (a) Intact plant (5-month-old) with first flowering. (b) Plantlets (5-month-old) cultured on MS+40 mg/L $ADSO_4$+2.0 mg/L NAA+1.0 mg/L BAP. (c) Plantlets (7-month-old) cultured on MS+40.0 mg/L $ADSO_4$+2.0 mg/L NAA+1.0 mg/L BAP. (d) Stunted plant (dwarf) after 3 weeks being acclimatized. (e) Nodule formation on roots after 6 weeks being acclimatized. (f) First twiner formation after 7 weeks being acclimatized. (g) Successful first flowering after 12 weeks being acclimatized. (h) Flower structure. (i) First fruiting or seeds pod.

6.3.2.1 Roots and Nodule Formation of *Clitoria ternatea* L.

After 6 weeks being acclimatized, nodules were formed at the roots of *C. ternatea* L. (Figure 6.2). Initially, 3 white nodules were observed attached to the roots. The white nodules became orange in colour after 2 weeks.

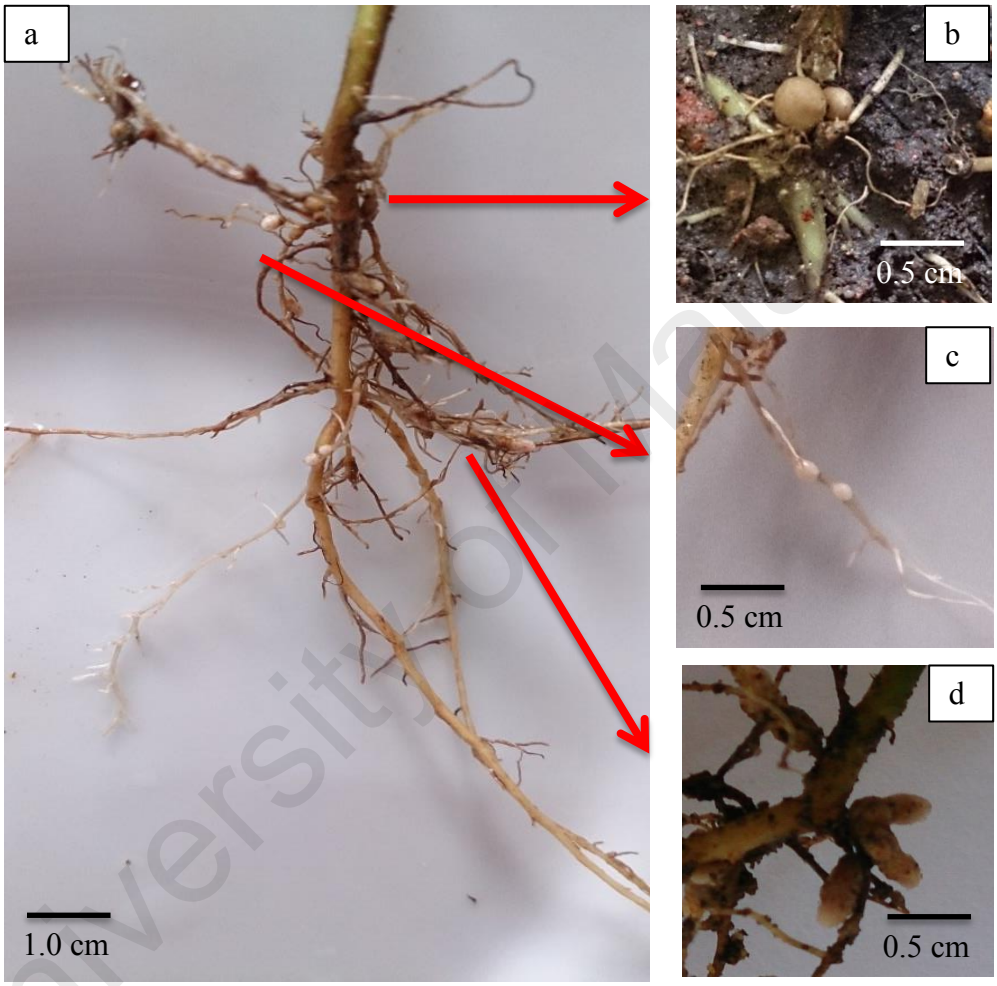


Figure 6.2 : Nodules formation on roots system of *Clitoria ternatea* L. after 8 weeks being acclimatized. (a) Structure of roots and nodules. (b) Orange coloured nodules. (c) White coloured and round shaped of nodules. (d) White coloured and oval shaped of nodules.

6.3.2.2 Flowering and Fruiting of *Clitoria ternatea* L.

After 12 weeks being acclimatized, the *ex vitro* plants started to flower (Figure 6.3) with 3-4 vivid blue coloured petals (3.2-4.5 cm). The vigorous growth of the plantlets with dark green pinnate leaves and shorter stem was observed. Even though, the twiner keeps growing, however no climbing activity was observed. The plants remained in 40-60 cm in stem height, which was much shorter as compared to *in vivo* plants of the same age (3-month-old). After 4-8 days, the flowers developed into flattened pea-like fruit (3.0-6.8 cm), followed by green seed pod formation (3-5 cm). A week after, the green fruit became matured seed pod (brown coloured) with five seeds. The *ex vitro* flowers were smaller (1.6-2.3 cm) but with thicker petals and darker vivid blue than *in vivo* flowers. Generally, the *ex vitro* plants were healthier and miniature with more vigorous growth, however, shorter in stem height compared to *in vivo* grown plants. The size, colour and quantity of pinnate leaves were higher as compared to *in vivo* plants. No flower obtained from *in vitro* grown plant.

Figure 6.3 : Morphology and flowering of *Clitoria ternatea* L. (a) No flowers formation in 10 weeks. (b) Vivid blue flowers formation after 12 weeks. (c) Morphology of leaves, flower and seeds.

6.3.3 Acclimatization and Macromorphology of *Onobrychis viciifolia* Scop.

After 7 months under *in vitro* condition, the plantlets of *O. viciifolia* Scop. were transferred to natural environment. Table 6.3 and Figure 6.4 showed detailed growth performance of *O. viciifolia* Scop., grown *in vivo*, *in vitro* and *ex vitro.* Overall, the *in vivo* and *ex vitro* plants only survived for 3-4 weeks, due to the poor adaptations to the high temperature (30±1ºC). The *in vivo* leaves showed darker green and larger 0.45±0.31) than *in vitro* (0.21±0.09) and *ex vitro* (0.27±0.07) grown plant, however with minimum number of leaves (3-6).

Although, the survival rate was higher (71.58±1.27) on topsoil at the beginning of the experiment, however, after 4 weeks the plants failed to adapt and became weak and died. The *in vitro* regenerated plants survived up to 8-10 months, whilst *ex vitro* only for one month.

Table 6.3 : The comparison of growth performances (stem, leaves, flowers and nodules) of *Onobrychis viciifolia* Scop., grown *in vivo*, *in vitro* and *ex vitro*.

Macromorphology	Observations		
	In vivo (1-month-old mother plant)	*In vitro* (7-month-old regenerated plant)	*Ex vitro* (1-month-old acclimatized plant)
A.VEGETATIVE SYSTEM			
Stems			
Description	Perennial legume with hollow stem.	Perennial legume with hollow stem.	Perennial legume with hollow stem.
Stem height (cm)	**8.42±0.47a**	6.98±0.85b	7.11±0.55ab
Leaves			
Description	Pinnate with 3 largest and darkest green leaflets in nearly triangular shape.	Pinnate with 3 large and dark green leaflets in nearly triangular shape.	Pinnate with 3 larger and darker green leaflets in nearly triangular shape.
Leaves width (cm)	**0.45±0.31a**	0.21±0.09b	0.27±0.07b
Roots			
Description	Large and deep tap root arising from a branching root crown.	Large and deep tap root.	Large and deep tap root.
Number of nodules	0	0	0
B.REPRODUCTIVE SYSTEM			
Flowers			
Description	Normally, flowers are pink, white or purple and tightly arranged in a compact raceme with tiny 20-50 flowers per head, each with its own banner, wings and keel. No flower obtained in the present study.	No flower obtained.	No flower obtained.
Number of flowers	0	0	0
Fruit pods			
Description	Normally, large single-seeded pod.	No fruit pod obtained.	No fruit pod obtained.
Fruit pod length (cm)	0	0	0

Mean values with different letters within a row are significantly different at p=0.05, by one way ANOVA and Duncan's Multiple Range Test (DMRT). n=30.

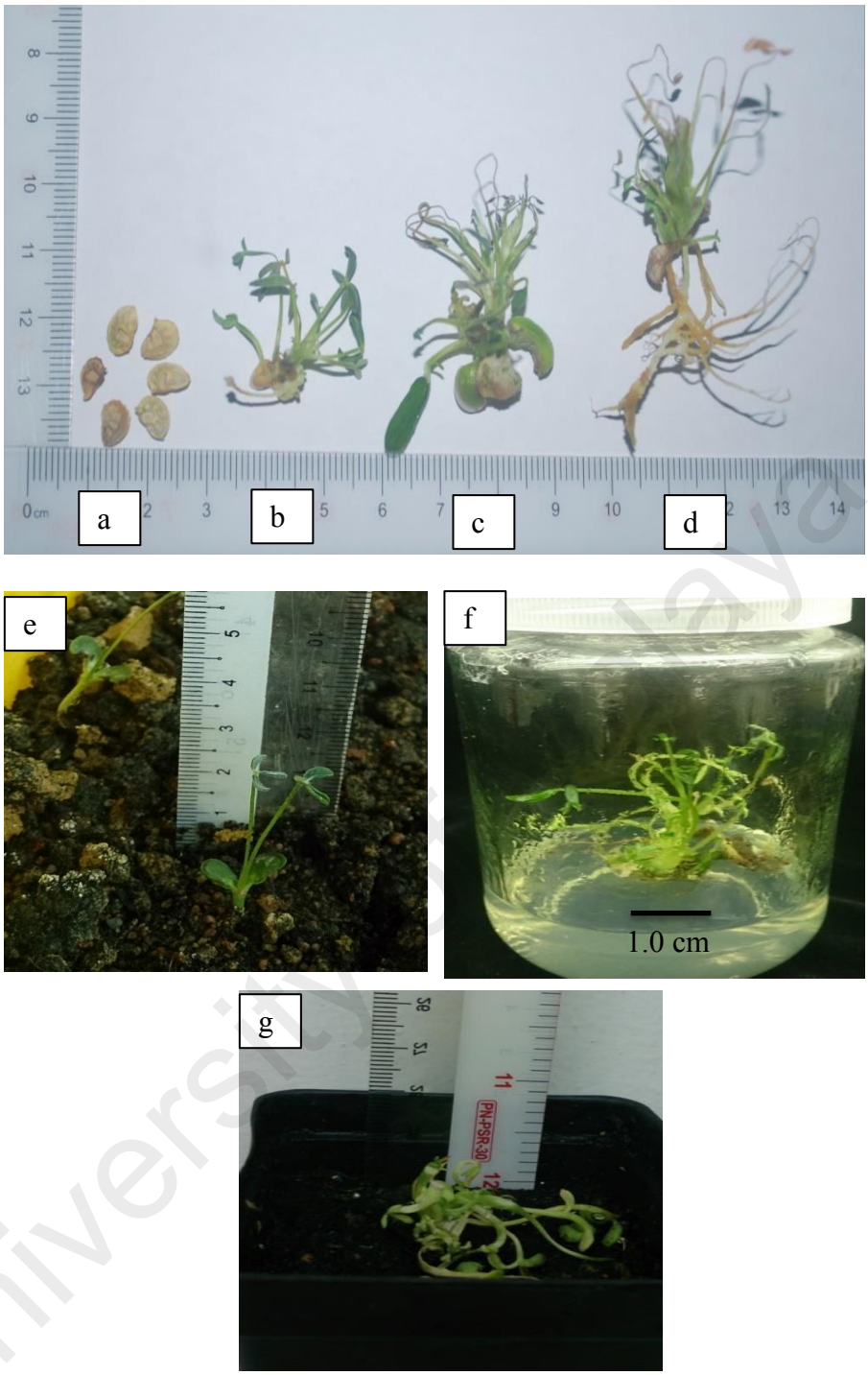


Figure 6.4 : Regeneration and acclimatization of *Onobrychis viciifolia* Scop.from root explant. (a) One seed (1-2 mm) in a large seed pod (0.3-0.5 mm). (b) Plantlet (4-month-old). (c) Plantlets (5-month-old) cultured on MS+40.0 mg/L $ADSO_4$+1.0 mg/L NAA+2.0 mg/L BAP. (d) Completed plantlet, 7-month-old. (e) *In vivo* grown plant, after 3 weeks germination. (f) Plantlets (7-month-old) cultured on MS+40.0 mg/L $ADSO_4$+1.0 mg/L NAA+2.0 mg/L BAP, after 7 months. (g) After 6 weeks being acclimatized.

6.3.4 The Root System of *Clitoria ternatea* L. and *Onobrychis viciifolia* Scop., Grown *In Vivo, In Vitro* and *Ex Vitro*

The root systems of both *C. ternatea* L. and *O. viciifolia* Scop. consist of tap root, primary roots and secondary roots. Nodules formed from these perennial legumes are finger-like in shape. In Leguminosae, the symbiotic association with N-fixing rhizobia bacteria which live in small growths attached to the plant roots called nodules. This formation of nodules functioning as biological nitrogen fixation which change unusable gas nitrogen (N_2) to ammonia (NH_3). All organisms use NH_3 (form of nitrogen) to manufacture amino acids, proteins, nucleic acids and other nitrogen-containing components. Initially, the comparison of growth performance especially in root formation and the original size of *C. ternatea* L. and *O. viciifolia* Scop. plants, cultured on on MS+40 mg/L $ADSO_4$ (control) was shown in Figure 6.5 and Figure 6.6. As observed in CHAPTER 3, *O. viciifolia* Scop. was shorter as compared to *C. ternatea* L. when germinated in MS media supplemented with hormones ($ADSO_4$, NAA, BAP, 2,4-D and KIN), which is the same as characteristic when seeds germinated on soil (*in vivo* plants). Generally, *C. ternatea* L. grew vigorously under *in vivo* condition, whilst, *O. viciifolia* Scop. performed better in *in vitro* condition (cultured on MS media with the addition of 40 mg/L $ADSO_4$), whereby more stems (10-19 stem) and three leaves for each stem was observed. Table 6.4 compared the root formation, grown *in vivo* and *in vitro* before being transferred to the soil. Unfortunately, the *ex vitro* plants of *O. viciifolia* Scop. only survived for 2-3 weeks after acclimatization. The plantlets became weak and died.

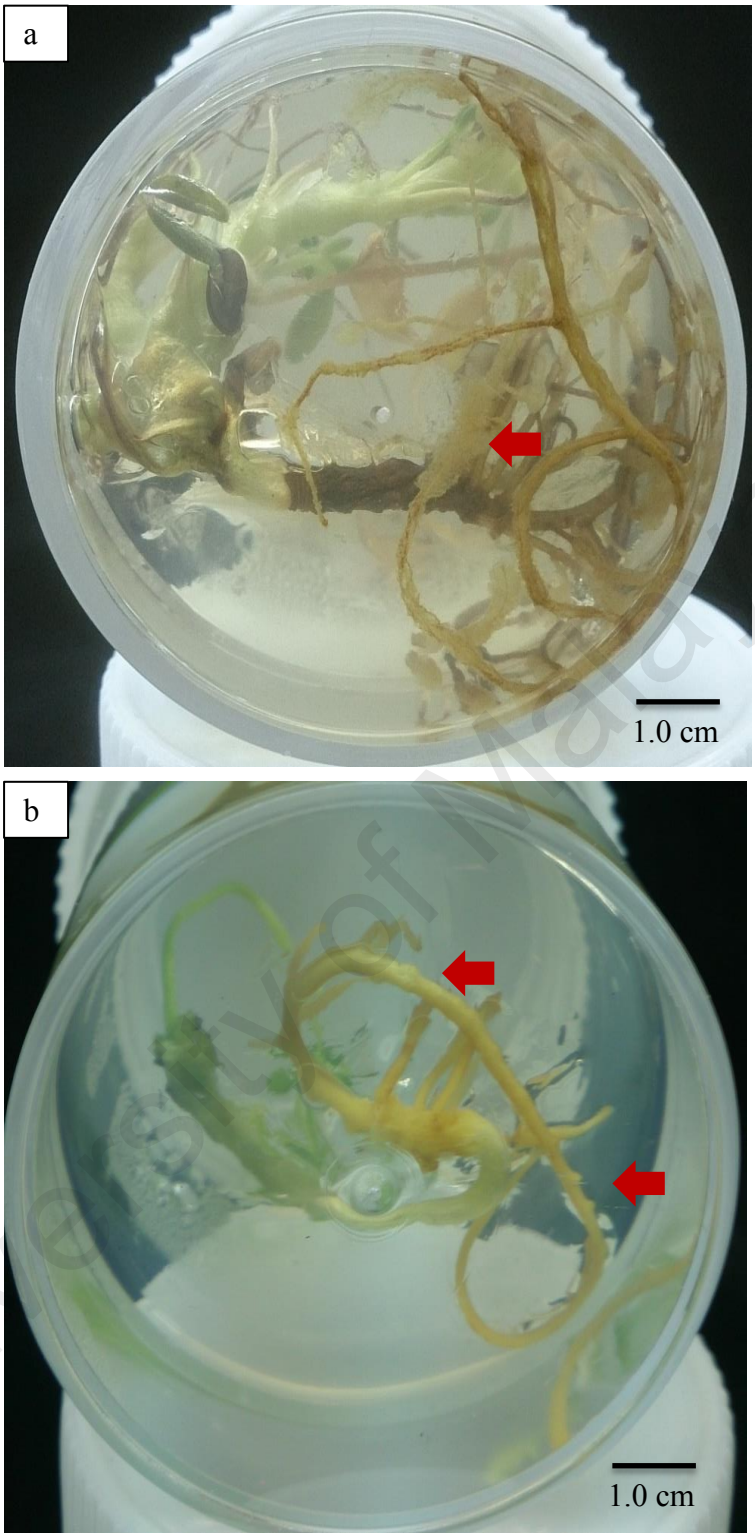


Figure 6.5 : Roots formation of *Onobrychis viciifolia* Scop. (a) Abnormal roots with white callus from seeds germination. (b) Normal roots with nodes from regenerated plants.

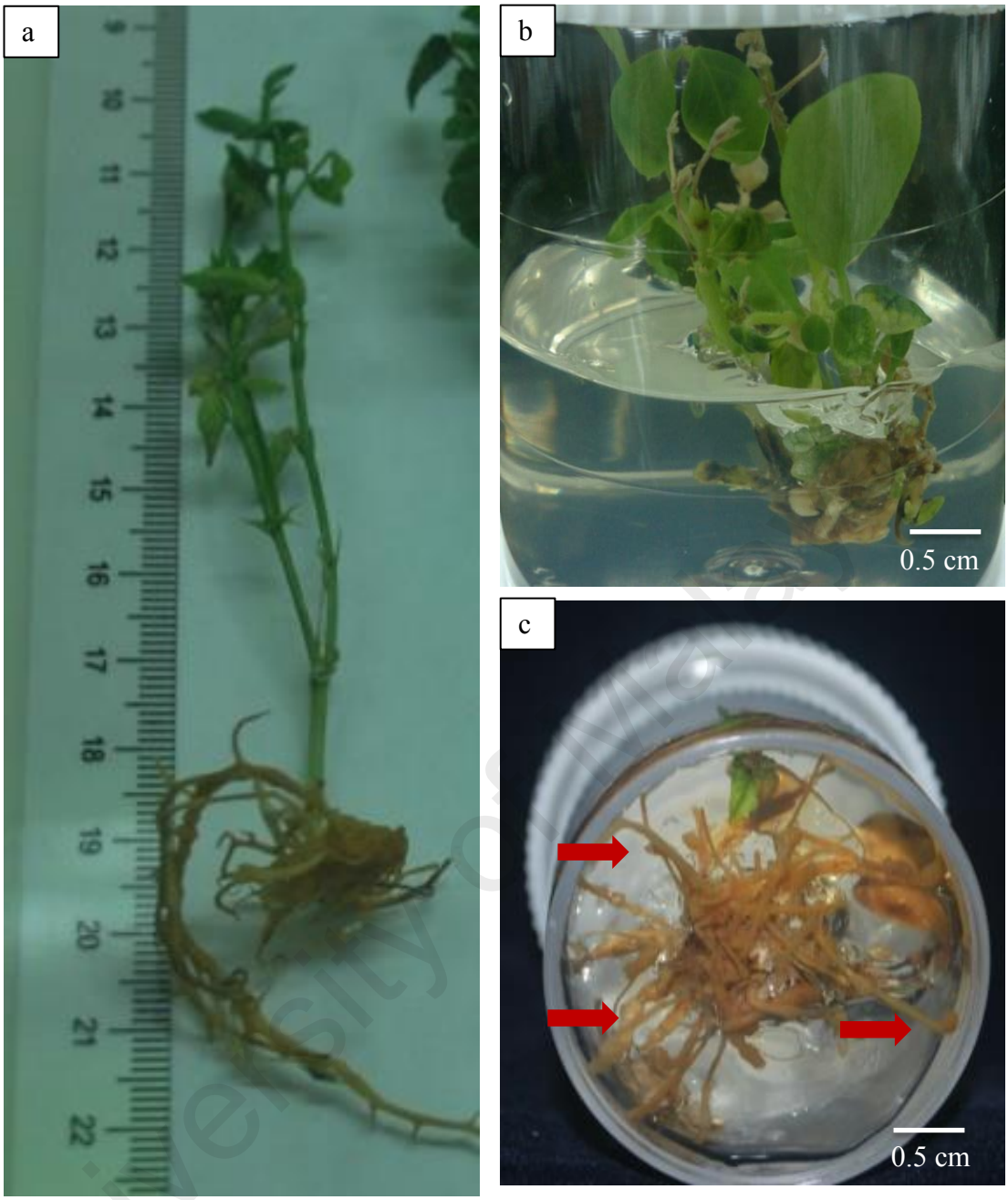


Figure 6.6 : Growth performances of *Clitoria ternatea* L. (a) Grown *in vivo* (1-month-old). (b) Multiple shoots, grown *in vitro* (4-month-old). (c) Roots with nodes, grown *in vitro* (6-month-old).

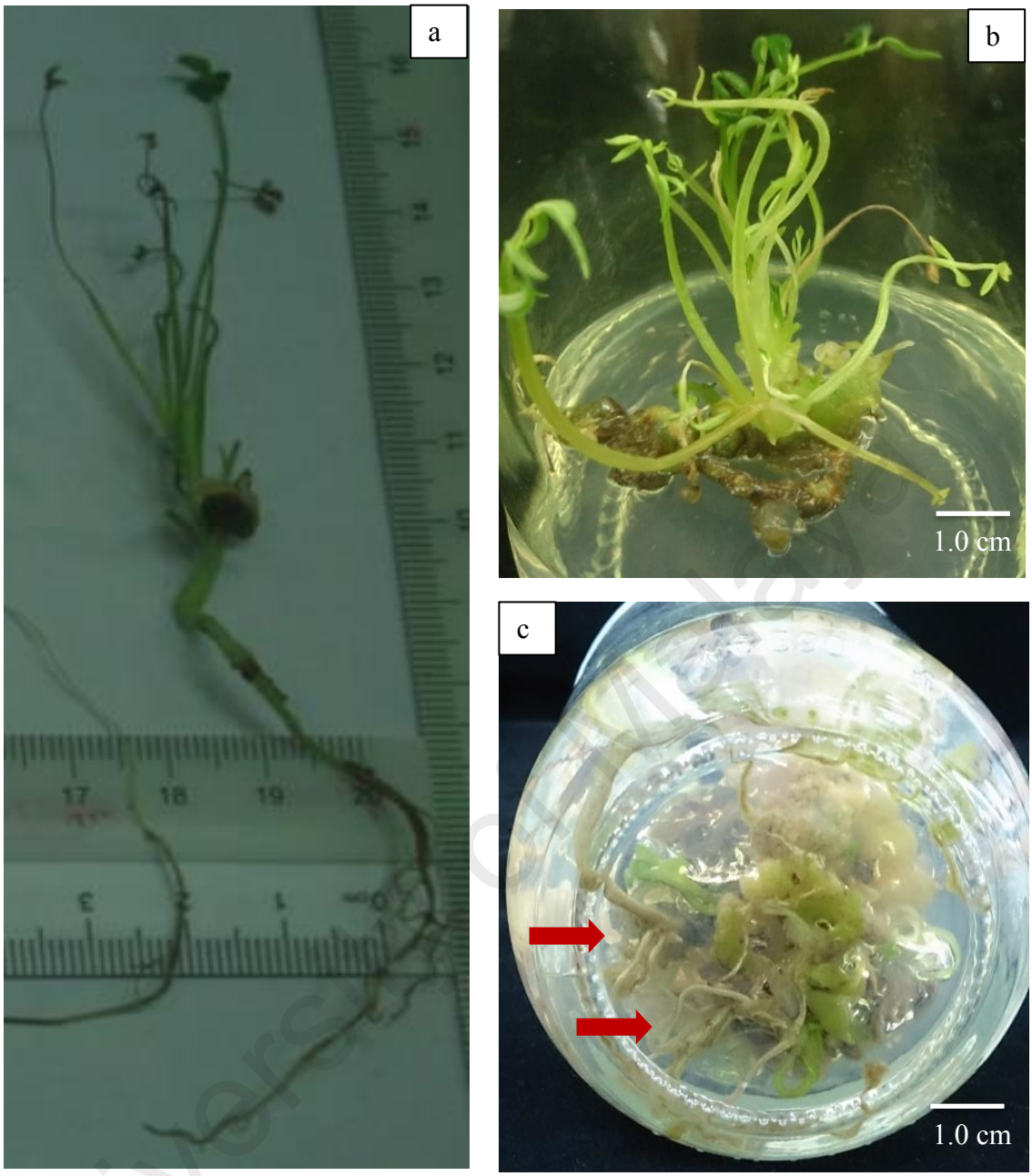


Figure 6.7 : Growth performances of *Onobrychis viciifolia* Scop. (a) Normal roots without nodes, grown *in vivo* (1-month-old). (b) Multiple shoots, grown *in vitro* (4 month-old). (c) Roots with nodes, grown *in vitro* (6-month-old).

6.4 SUMMARY OF RESULTS

1. Based on the survival rates on selected soils in Malaysia, plantlets of *C. ternatea* L. (64.71-91.07%) performed better than *O. viciifolia* Scop. (39.22-71.58%), with the highest survival rate at 91.07±0.96% and 71.58±1.27%, respectively.

2. After 7 months being acclimatized, the *ex vitro* plants of *C. ternatea* L. was shorter (39.16±0.28 cm), which was about half as compared to the *in vivo* plant height (95.03±0.83**)** , but the quality of leaves (2.11±0.11 cm) and flowers (9-16 flowers per plant) were better, as well as nodules formation (25-40 nodules per plant).

3. After 7 months of acclimatization in the green house, the regenerants of *C. ternatea* L. showed similar growth characteristics as the mother plants, whilst, *O. viciifolia* Scop. died after 2 months exposed to Malaysian temperature (30±1ºC).

4. *C. ternatea* L. (at age of 7-month-old), *in vivo* plants showed the highest stem (95.03±0.83 cm**)**, followed by *ex vitro* plants (39.16±0.28 cm), which was about half as compared to the *in vivo* plant height, however, the quality of leaves (2.11±0.11 cm) and flowers (9-16 flowers per plant) were the best among the three growth conditions (*in vivo*, *in vitro* and *ex vitro*).

5. Overall, the *in vivo* and *ex vitro* plants only survived for 3-4 weeks, due to the poor adaptations to the high temperature (30±1ºC). The *in vivo* leaves showed darker green and larger (0.45±0.31) than *in vitro* (0.21±0.09) and *ex vitro* (0.27±0.07) grown plant, however with minimum number of leaves (3-6).

6. Generally, *C. ternatea* L. grew vigorously under *in vivo* condition, whilst, *O. viciifolia* Scop. performed better in *in vitro* condition (cultured on MS media with the addition of 40 mg/L $ADSO_4$), whereby more stems (10-19 stem) and three leaves for each stem was observed.

CHAPTER 7

MICROMORPHOLOGICAL STUDIES THROUGH FESEM AND HISTOLOGY ANALYSIS OF *Clitoria ternatea* L. AND *Onobrychis viciifolia* Scop., GROWN *IN VIVO* AND *IN VITRO*

7.1 EXPERIMENTAL AIMS

The tropical legume (*Clitoria ternatea* L.) and temperate legume *(Onobrychis viciifolia* Scop.) are grown as protein source for animal feed, particularly in organic farming. The growth performances of these species are influenced by temperatures and climatic regions. Growth performance can be measured from leaf, stem, flower and fruit production.

The importance of micromorphological features are for the taxonomic consideration or identification (Parveen et al., 2000) and micromorphological aspects of the leaf surface are influenced by habitat, as well as the exposure of sunlight for adaptation. According to Kathiresan et al. (2011), the micromorphological parameters of different plant parts have been used as aids in the taxonomical recognition of species. The foliar epidermis (including leaf), is one of the most noteworthy taxonomic characters from a biosystematics point of view. Size, distribution, and frequency of stomata have been found to be specific to taxa and are used as significant parameters in taxonomy as well as in elucidating phylogeny (Ahmed, 1979; Rajagopal, 1979; Idu et. al., 2000; Barkatullah et. al., 2014). Characteristics, distribution and and frequency of stomata have been found to be specific to some taxa and are used as significant micromorphology in taxonomy as well as phylogeny (Rajagopal, 1979).

Based on the results in CHAPTER 6, the acclimatization and adaptation of the regenerants were successful for *C. ternatea* (91%) and *O. viciifolia* (71%), after 7 months under *in vitro* condition. Due to the main advantages of *C. ternatea* L. (high-

protein forage crop) and *O. viciifolia* Scop. (anti-bloating forage crop), these plants can be potentially introduced and cultivated in Malaysia for domestic livestock industry.

The objectives of this specific chapter were:

1. To identify and compare the stomata and trichomes distribution and ultrastructural features from leaves of *C. ternatea* L. and *O. viciifolia* Scop., grown *in vivo* and *in vitro*, using Field Emission Scanning Electron Microscope (FESEM).

2. To investigate the histology and anatomy (cuticle, palisade mesophyll, spongy mesophyll, vascular bundle, air, space, stomata and guard cell) from leaves of *C. ternatea* L. and *O. viciifolia* Scop., grown *in vivo* and *in vitro*.

7.2 MATERIALS AND METHODS

7.2.1 Plant Materials

The acclimatization of the regenerants (in CHAPTER 6) derived from root explant resulted different survival rates of *C. ternatea* L. and *O. viciifolia* Scop., 64.71-91.07% and 39.22-71.58%, respectively. For further understanding of the micromorphological features and structures, 7-month-old leaf samples from *in vivo* (grown on soil) and *in vitro* (grown on MS media supplemented with $ADSO_4$, NAA and BAP) plants were examined and compared using Field Emission Scanning Electron Microscope (FESEM). The *in vitro* leaf samples were obtained from results in CHAPTER 3, which were the optimum media for regeneration of *C. ternatea* L. with the highest root formation (8.72±1.27) and shoots (12.03±0.12) formation was obtained from root explant that were cultured on MS supplemented with combinations of 40.0 mg/L $ADSO_4$, 2.0 mg/L NAA and 1.0 mg/L BAP, while, the highest shoots (17.97±0.09) and roots (4.11±0.42) formation of *O. viciifolia* Scop. was obtained from root explant cultured on MS supplemented with 40.0 mg/L $ADSO_4$, 1.0 mg/L 2,4-D and 3.0 mg/L KIN.

7.2.2 Morphology and Anatomy

7.2.2.1 Field Emission Scanning Electron Microscope (FESEM) Examinations on Leaves of *Clitoria ternatea* L. and *Onobrychis viciifolia* Scop., Grown *In Vivo* and *In Vitro*

The young leaves (5-month-old) obtained from in vitro system were compared with intact plants (in vivo). Standard procedures were followed for field Emission Scanning Electron Microscope, FESEM (FEG Quanta^{TM} 450, EDX-OXFORD FE-SEM). A section of 3 mm^2 of fresh young leaves (both adaxial and abaxial surfaces) was fixed on to double side adhesive tape on labelled stubs. For optimal resolution, the detector used was Large Field Low vacuum SED (LFD) while the working distance used were at 8.5 to 9.6 mm from the final lens (microscope), at low

accelerating voltage (5.00 kV). The visuals of trichomes and stomata were captured and analyzed (distribution and structure) on both upper (adaxial) and lower (abaxial) surfaces.

7.2.2.2 Histology Study on Leaves of *Clitoria ternatea* L. and *Onobrychis viciifolia* Scop., Grown *In Vivo* and *In Vitro*

Histology is the microscopic anatomic study of cells and tissue structure of plants or animals by using staining method. One of the microscopic approach utilizes techniques is light microscope which is to analyze characteristics of presence and absence of trichomes (hairs), oil glands, canals, particular cell types, seed or pollen morphology and vascular traces. In the present study, *in vivo* and *in vitro* grown plants (5-month-old leaf explants)were fixed in FAA solution (9:1:1 of 70% ethyl alcohol:glacial alcohol:formaldehyde). Then, the samples were washed three times with 70% alcohol and dehydrated in 90% v/v tertiary butyl alcohol (TBA). The samples were then infiltrated in mixture of paraffin oil and TBA and kept overnight. After that, the samples were placed into melted paraffin wax (49ºC) for overnight and transfered into melted paraffin wax (56ºC) for 24 hours. Embedding process was done by pouring 56ºC of paraffin wax on the paper boat. Then, the samples were sectioned (8 µM) with rotary microtome (Leica, Germany) stained with safranin 'O' and Alcian Blue and mounted on the slides using albumin. Finally, the samples were viewed under Axioskop Zeiss (GERMANY) microscope attached to AxioCam Mrc camera and then analyzed using Axio vision 4.7 software.

7.2.3 Data Analysis

Data and figures were analyzed by Field Emission Scanning Electron Microscope, FESEM (FEG Quanta^{TM} 450, EDX-OXFORD FE-SEM) and Axio vision 4.7 software (Axioskop Zeiss GERMANY microscope).

7.3 RESULTS

7.3.1Comparison of FESEM Analysis between *Clitoria ternatea* L. and *Onobrychis viciifolia* Scop.

FESEM technology revealed the presence of non-glandular trichomes with sharp-point end were scattered over the leaf surfaces. Most of the non- glandular trichomes on *in vitro* leaves appeared creased and wrinkled on adaxial surface. FESEM micrographs showed the distribution of stomata and non-glandular trichomes from both surfaces (adaxial and abaxial) of *C. ternatea* L. leaves, grown *in vivo* (Figure 7.1) and *in vitro* (Figure 7.2), with higher stomata and trichome number on abaxial surface of *in vitro* grown leaves. FESEM micrographs showed the distribution of stomata and non-glandular trichomes from both surfaces (adaxial and abaxial) of *O. viciifolia* Scop. leaves, grown *in vivo* (Figure 7.3) and *in vitro* (Figure 7.4), with higher stomata and trichome number on abaxial surface of *in vitro* grown leaves. Trichomes (hairs) are unicellular or multicellular outgrowths that originate from the aerial epidermis and vary in morphological features, location and mode of secretion. Glandular trichomes are associated with the production of chemicals that provide defense against herbivores and pathogens. It has been suggested that non-glandular trichomes serve various functions in plants, including reducing the heat load, reflectance of UV light, provide protection from insects and herbivores, increase tolerance to freezing and maintain water balance in leaves. The non-glandular trichome is supported by a basal cellular pedestal. It has been reported that the basal cellular pedestal provides mechanical support and serves as a point for the attachment of trichomes to the epidermis. The stalk of the non-glandular trichome is densely covered with cuticular warts, which could be indicative of leaf maturity and which may be involved in helping the hairs stay free of dust by promoting cleaning during rainfall; the so called 'Lotus effect'. The density of non

glandular trichomes is an adaptation which used to limit incoming UV light and thus protect vascular tissues in leaf.

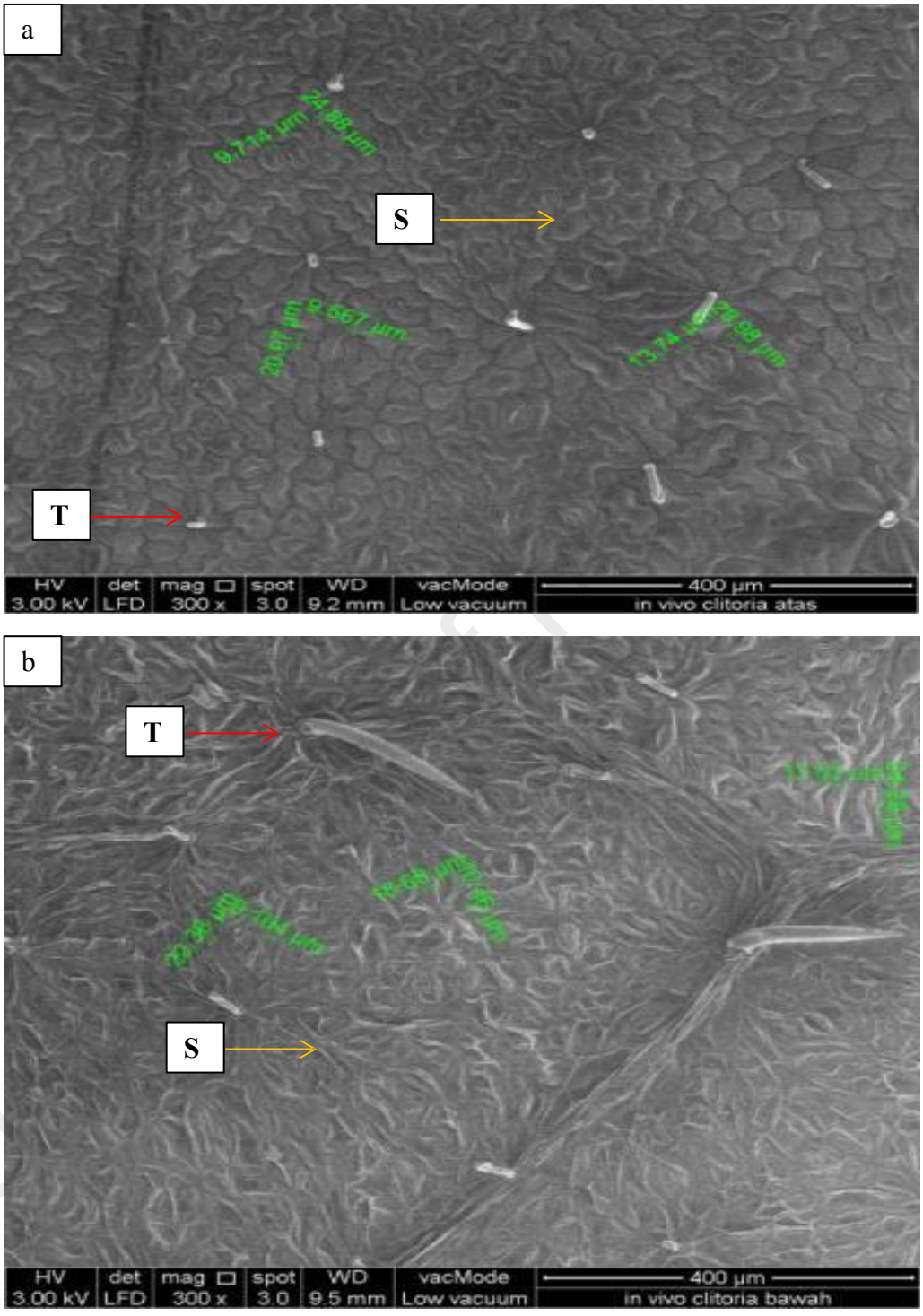


Figure 7.1 : Field Emission Scanning Electron Microscope (FESEM) micrographs showing the distribution of stomata and non-glandular trichomes from both surfaces (adaxial and abaxial) of *Clitoria ternatea* L. leaves, grown *in vivo*, viewed at 300x magnification. (a) Adaxial surface of *in vivo* grown leaf. (b) Abaxial surface of *in vivo* grown leaf.

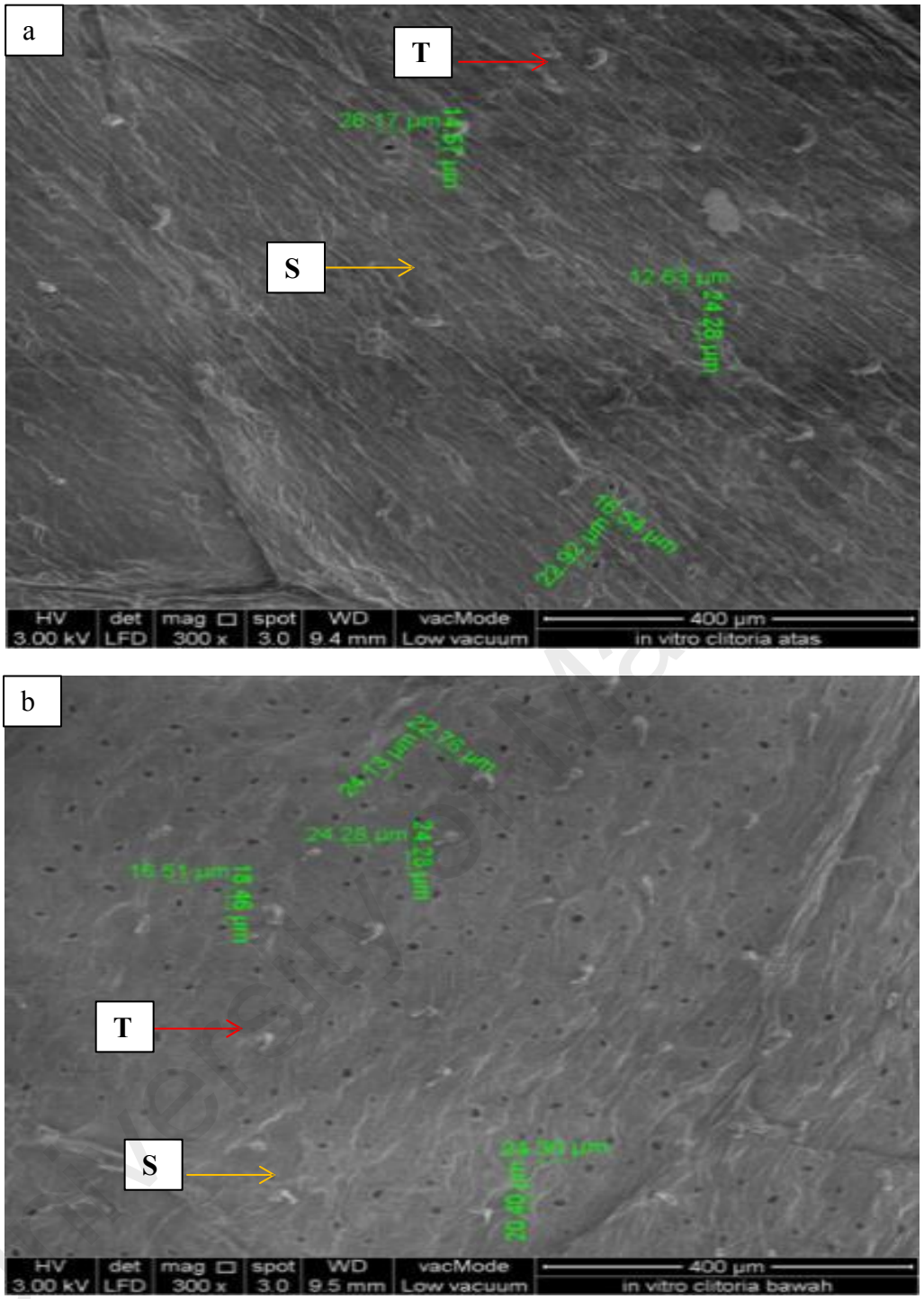


Figure 7.2 : Field Emission Scanning Electron Microscope (FESEM) micrographs showing the distribution of stomata and non-glandular trichomes from both surfaces (adaxial and abaxial) of *Clitoria ternatea* L. leaves, grown *in vitro*, viewed at 300x magnification. (a) Adaxial surface of *in vitro* grown leaf. (b) Abaxial surface of *in vitro* grown leaf.

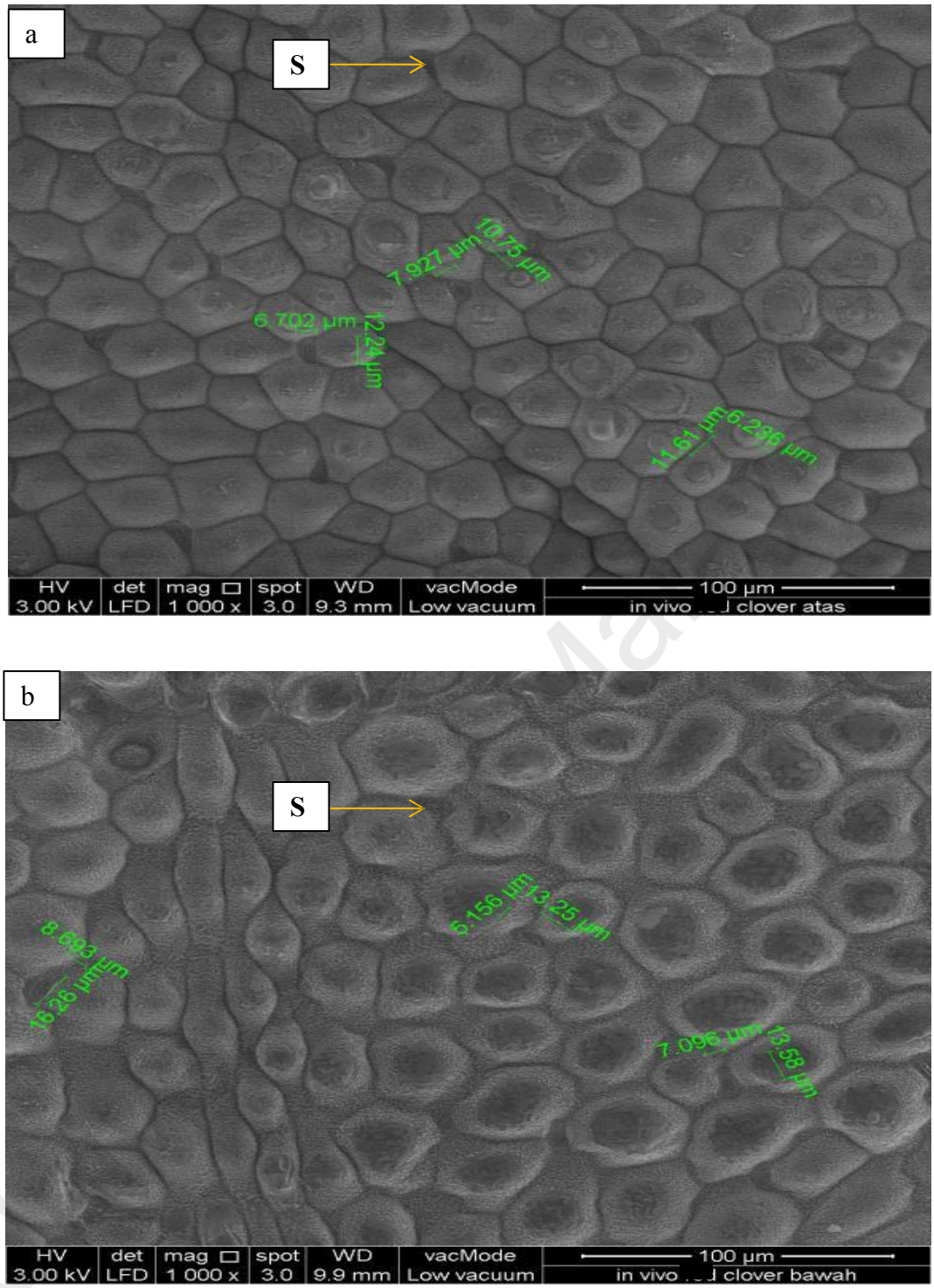


Figure 7.3 : Field Emission Scanning Electron Microscope (FESEM) micrographs showing the distribution of stomata from both surfaces (adaxial and abaxial) of *Onobrychis viciifolia* Scop. leaves, grown *in vivo*, no non-glandular trichomes (long-stalked capitate) were observed, viewed at 300x magnification. (a) Adaxial surface of *in vivo* grown leaf. (b) Adaxial surface of *in vivo* grown leaf.

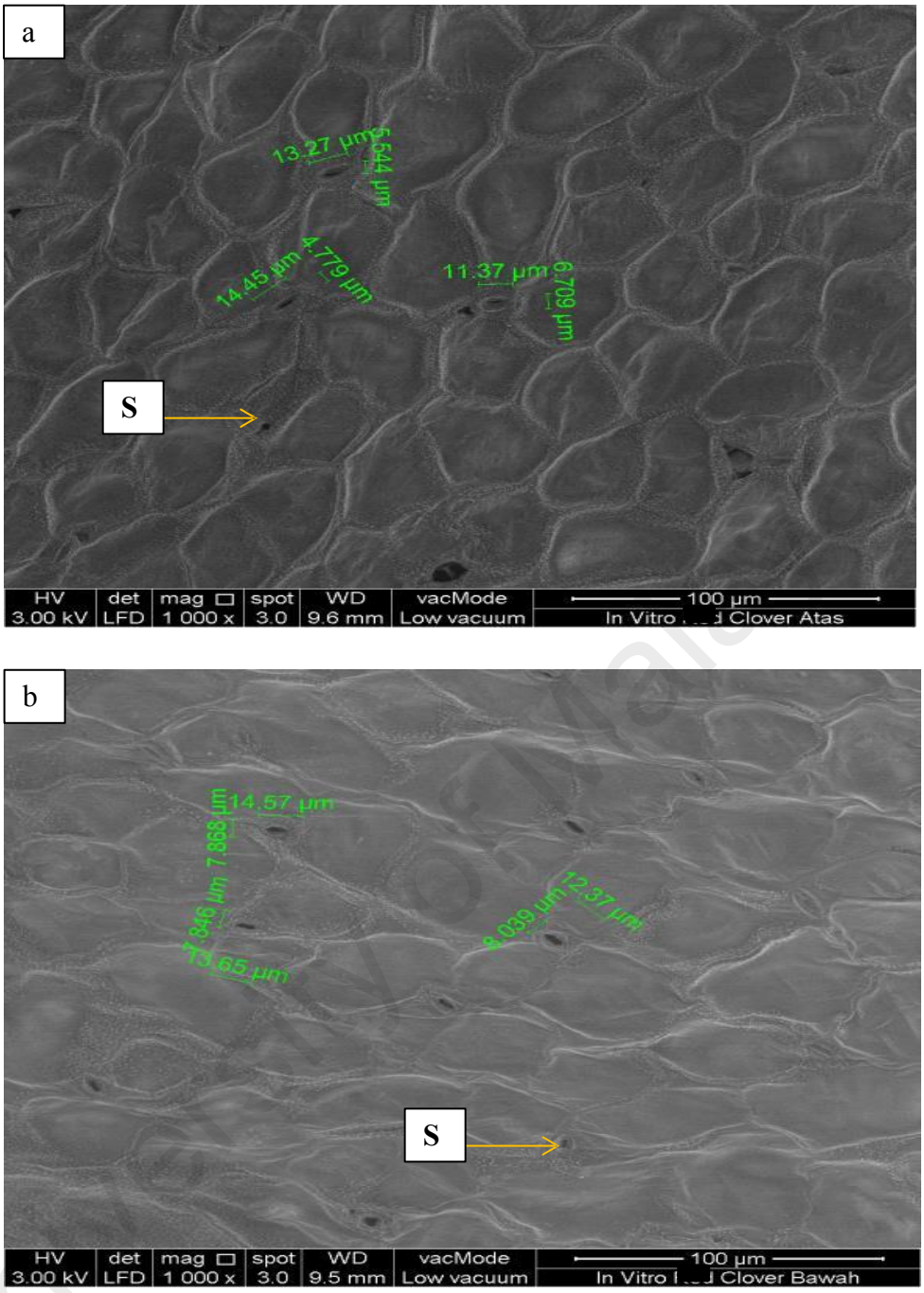
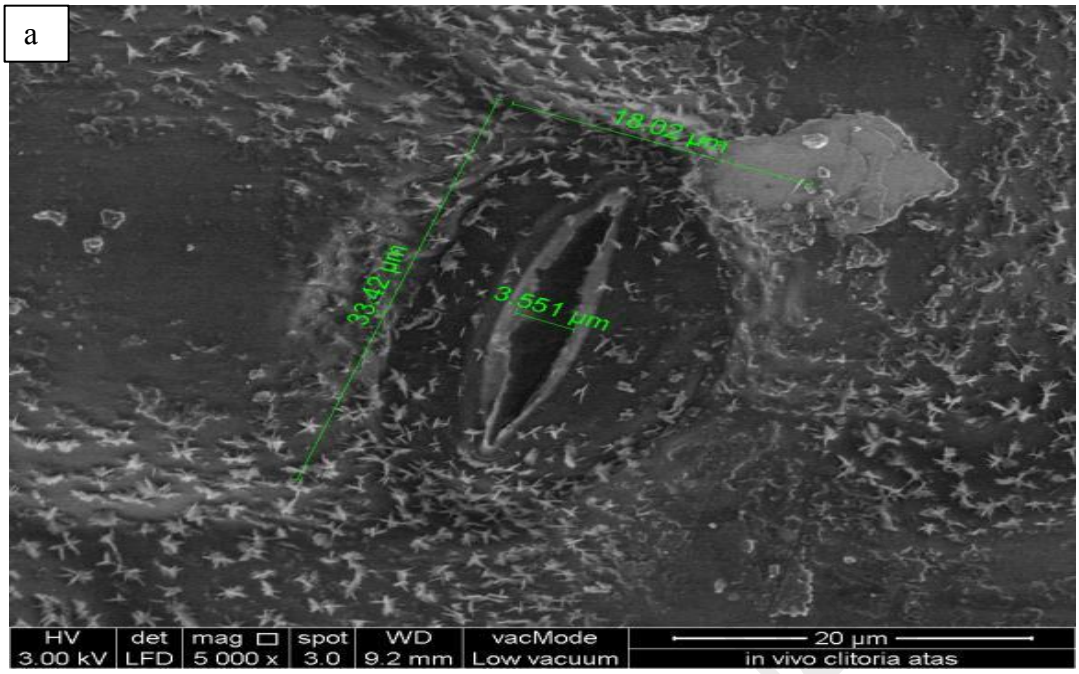


Figure 7.4 : Field Emission Scanning Electron Microscope (FESEM) micrographs showing the distribution of stomata from both surfaces (adaxial and abaxial) of *Onobrychis viciifolia* Scop. leaves, grown *in vitro*, no non-glandular trichomes (long-stalked capitate) were observed, viewed at 300x magnification. (a) Adaxial surface of *in vitro* grown leaf. (b) Adaxial surface of *in vitro* grown leaf.

The FESEM observation from *in vivo* (Figure 7.5) and *in vitro* (Figure 7.6) grown leaves of *C. ternatea* revealed that the stomata were in a shallow pit, this helps to reduce wind speed across the stomata and therefore slow transpirational water losses (Figure 7.3), while, the FESEM micrographs of *O. viciifolia* Scop. *in vivo* (Figure 7.7) and *in vitro* (Figure 7.8) grown leaves showed the close up of stomata from both surfaces (adaxial and abaxial) with no non-glandular trichomes (long-stalked capitate). Generally, for both species, the abaxial surface of the leaf had more stomata as compared to adaxial surface. Moreover, the results showed *in vitro* grown leaves obtained more stomata than *in vivo* leaves. The surface of in vivo leaf was smooth, whilst the *in vitro* appeared wrinkles. The micrographs also showed the appearance of hairy structure on both surfaces. The abaxial surface showed more hairy structure compared to adaxial surface. More hairy structure was observed in *C. ternatea* L. as compared to *O. viciifolia* Scop. The rough texture of leaf is caused by the presence of wax fibres (waxy cuticle). Stomata are small openings found in epidermis of green aerial parts, especially leaves of the plants. Usually, stomata are anomocytic type, present on the upper (adaxial) and lower (abaxial) surfaces of the leaves. The *in vivo* grown leaves of *C. ternatea* L. and *O. viciifolia* Scop. had more stomata compared to *in vitro* grown leaves. The stomata into different types on the basis of structure and shape of neighbouring epidermal or subsidiary cells, which helped in performing physiological functions like photosynthesis, respiration and transpiration usually present on both surfaces of the leaves.



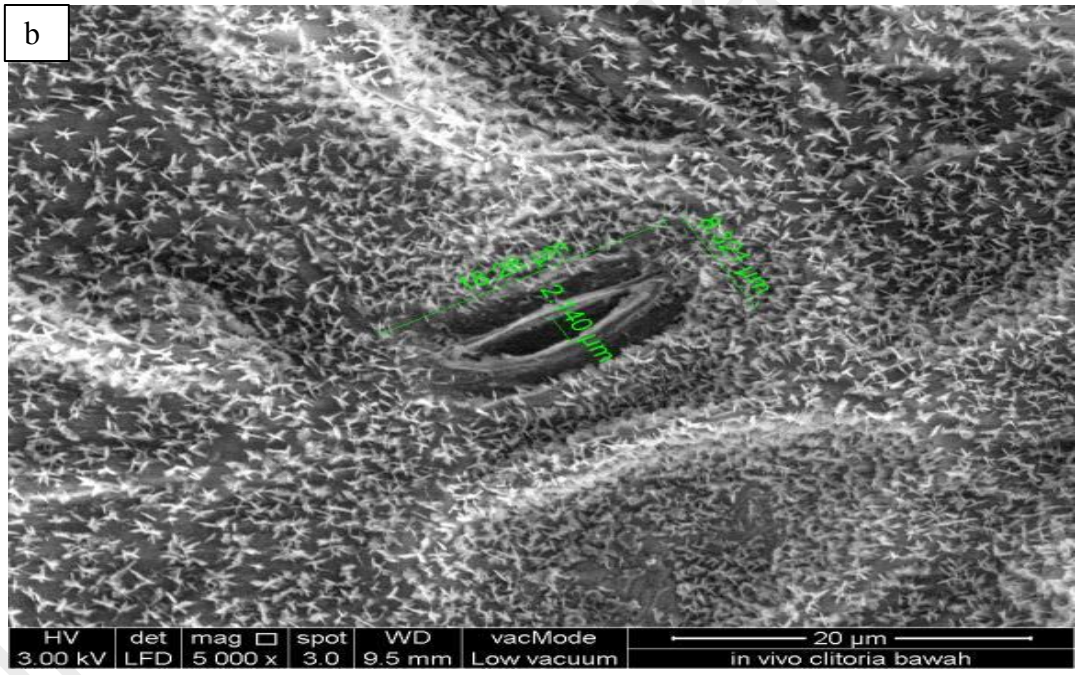


Figure 7.5 : Field Emission Scanning Electron Microscope (FESEM) micrographs showing the close up of stomata from both surfaces (adaxial and abaxial) of *Clitoria ternatea* L. leaves, grown *in vivo*, no non-glandular trichomes (long-stalked capitate) were observed, viewed at 300x magnification. (a) adaxial surface of *in vivo* grown leaf. (b) Adaxial surface of *in vivo* grown leaf.

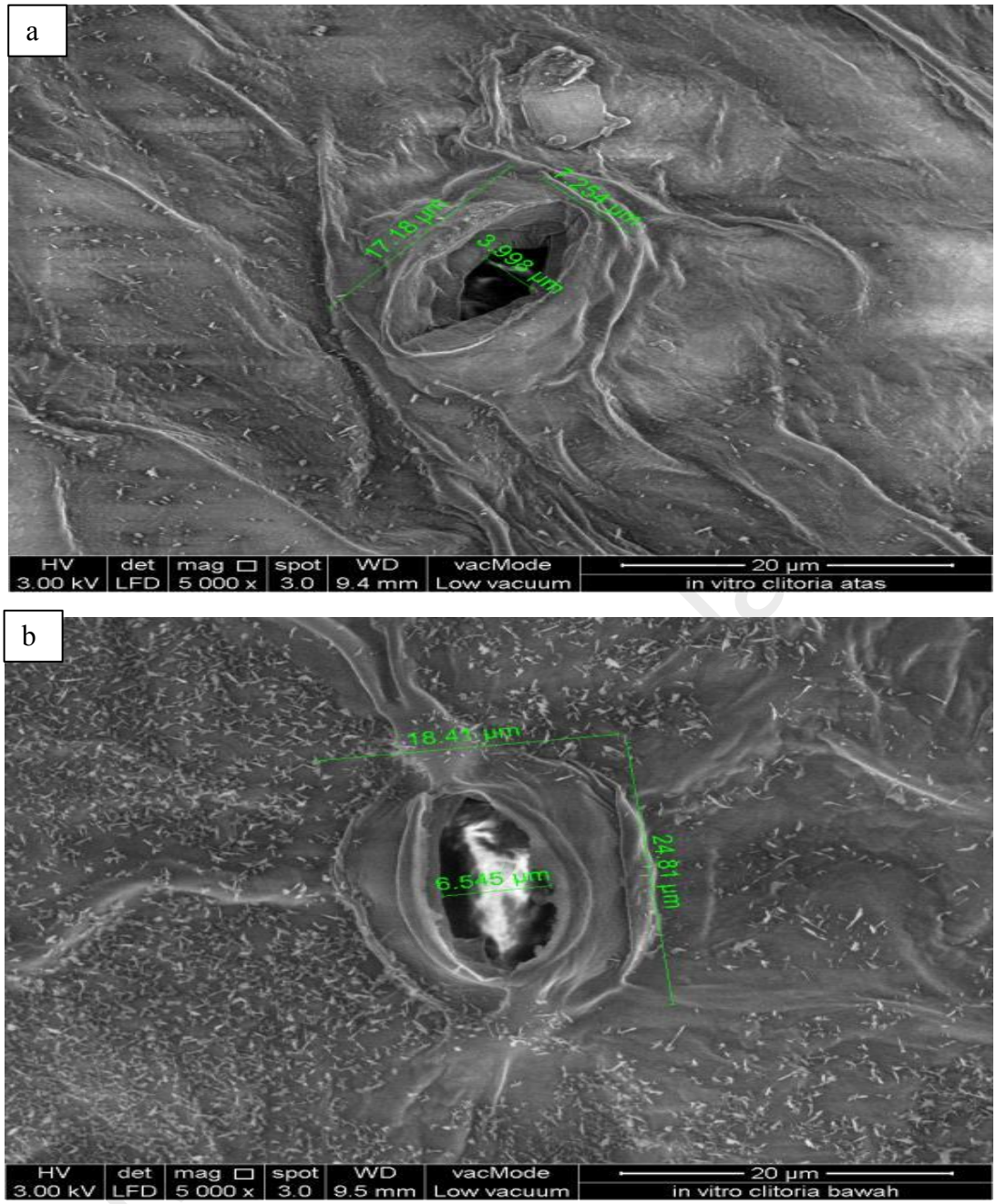


Figure 7.6 : Field Emission Scanning Electron Microscope (FESEM) micrographs showing the close up of stomata from both surfaces (adaxial and abaxial) of *Clitoria ternatea* L. leaves, grown *in vitro*, no non-glandular trichomes (long-stalked capitate) were observed, viewed at 300x magnification. (a) adaxial surface of *in vitro* grown leaf. (b) Adaxial surface of *in vitro* grown leaf.

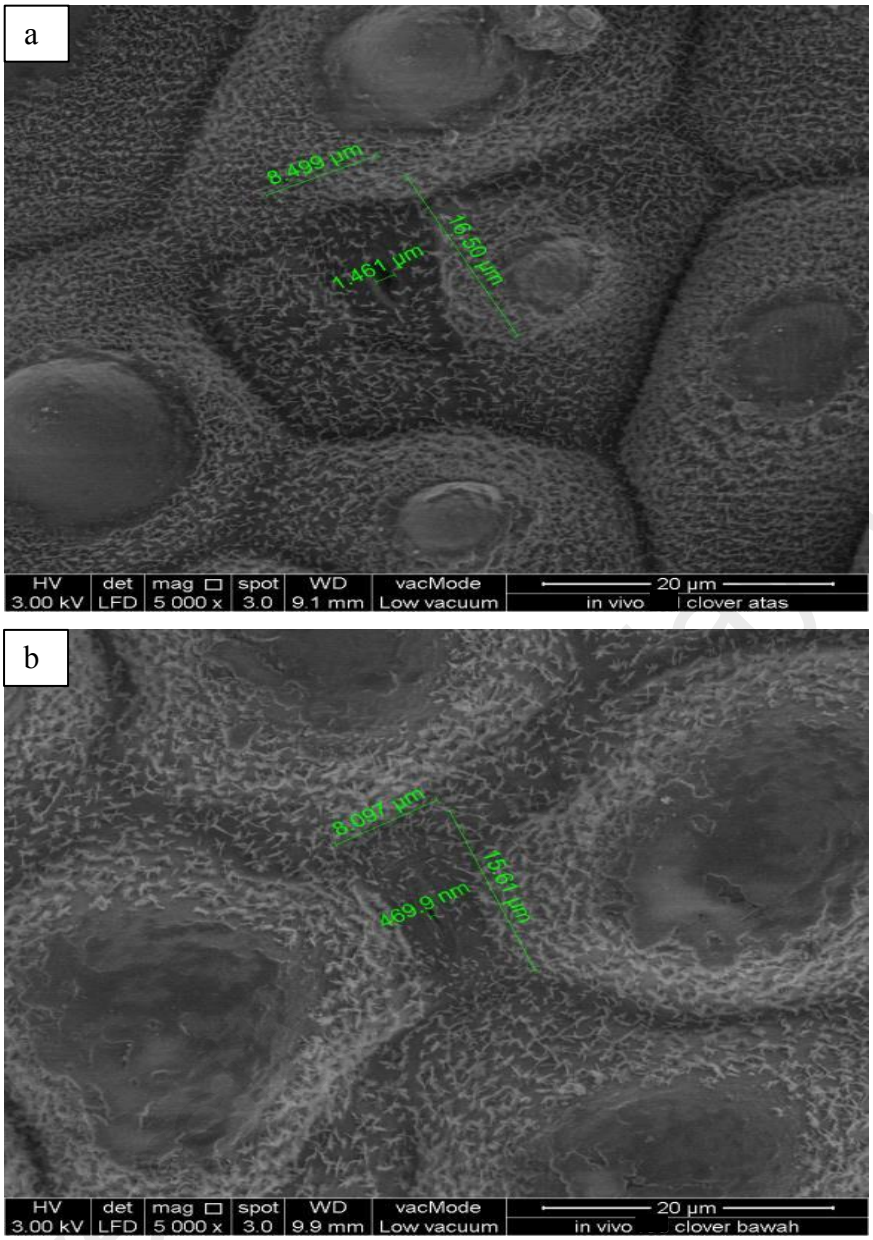


Figure 7.7 : Field Emission Scanning Electron Microscope (FESEM) micrographs showing the close up of stomata from both surfaces (adaxial and abaxial) of *Onobrychis viciifolia* Scop. leaves, grown *in vivo*, non-glandular trichomes (long-stalked capitate) were observed, viewed at 300x magnification. (a) Adaxial surface of *in vivo* grown leaf. (b) Adaxial surface of *in vivo* grown leaf.

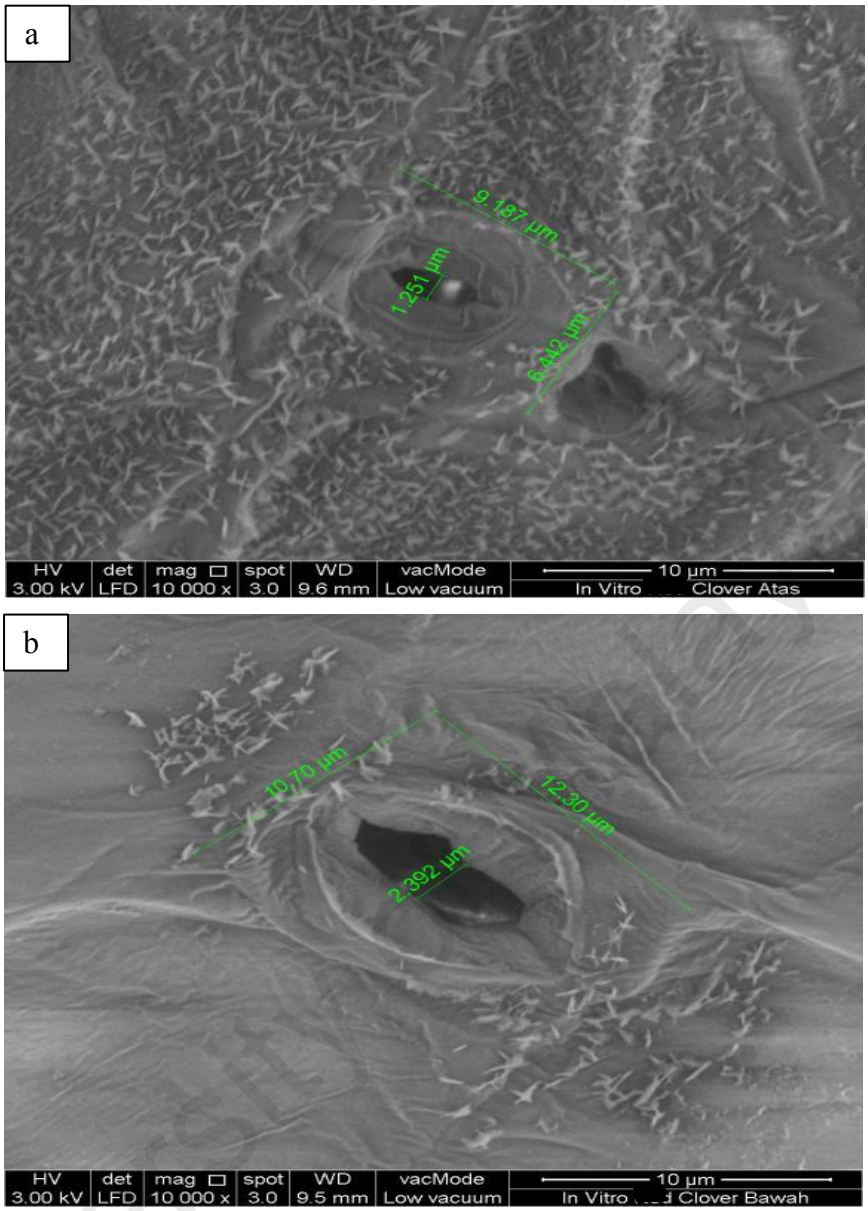


Figure 7.8 : Field Emission Scanning Electron Microscope (FESEM) micrographs showing the close up of stomata from both surfaces (adaxial and abaxial) of *Onobrychis viciifolia* Scop. leaves, grown *in vitro*, non-glandular trichomes (long-stalked capitate) were observed, viewed at 300x magnification. (a) Adaxial surface of *in vitro* grown leaf. (b) Adaxial surface of *in vitro* grown leaf.

7.3.2 Comparison of Histology Analysis between *Clitoria ternatea* L. and *Onobrychis viciifolia* Scop., Grown *In Vivo* and *In Vitro*

The Figures 7.9 and 7.10 showed the histology analysis of the ultrastructural features from leaf of *C. ternatea* L. and *O. viciifolia* Scop. grown *in vivo* and *in vitro* after 5 months, respectively. The adaxial (upper epidermis) from *in vivo* grown leaf of both species showed the presence of cuticle which affected by the sun exposure, besides providing protection against desiccation and pathogens. The clearer open and closed stomata were observed at abaxial (lower epidermis) which control the exchange of gases and water vapor between the leaf cells and the atmosphere. The stomatal organization consists of two subsidiary cells, one on each side of the guard cell pair.

Beneath the adaxial is a layer of vertically elongated palisade mesophyll cells (PM), which packed with chloroplasts (small circular bulges within many cells) and large air space. The lower half of the leaf contains the spongy mesophyll which loosely arranged network of cells of irregular shape and large air space. Overall, the ultrastructural features of *in vivo* leaf showed more air space in palisade and spongy mesophyll layer, while the *in vitro* leaf with compact cells.

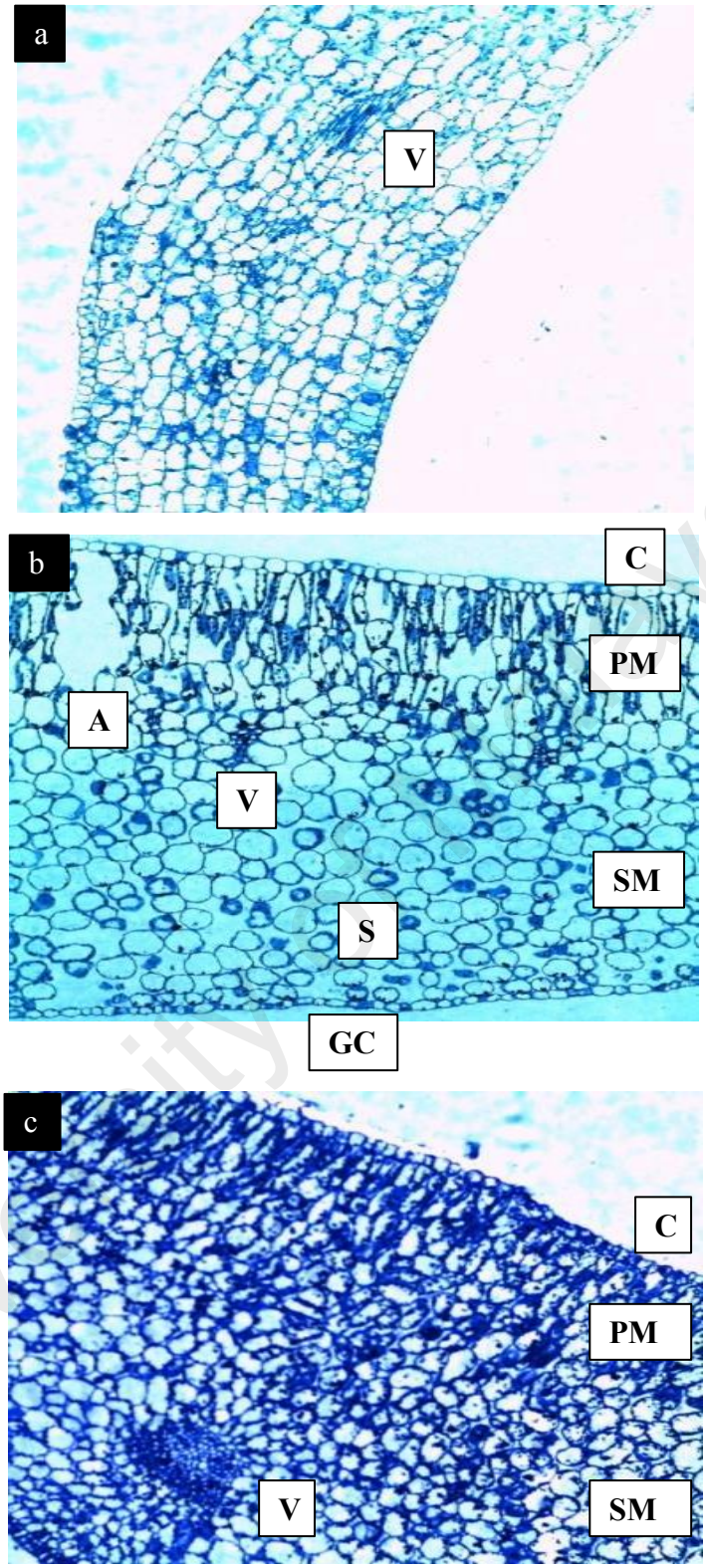


Figure 7.9: Light micrographs from leaves of *Clitoria ternatea* L. (5 months old) showing morphology and anatomy of cuticle (C) , palisade mesophyll (PM), spongy mesophyll (SM), vascular bundle (V), stoma (S), guard cell (GC) and air space (A), viewed at 40x magnification. (a) Longitudinal section of *in vivo* grown plant. (b) Cross section of *in vivo* plant. (c) Cross section of *in vitro* plant.

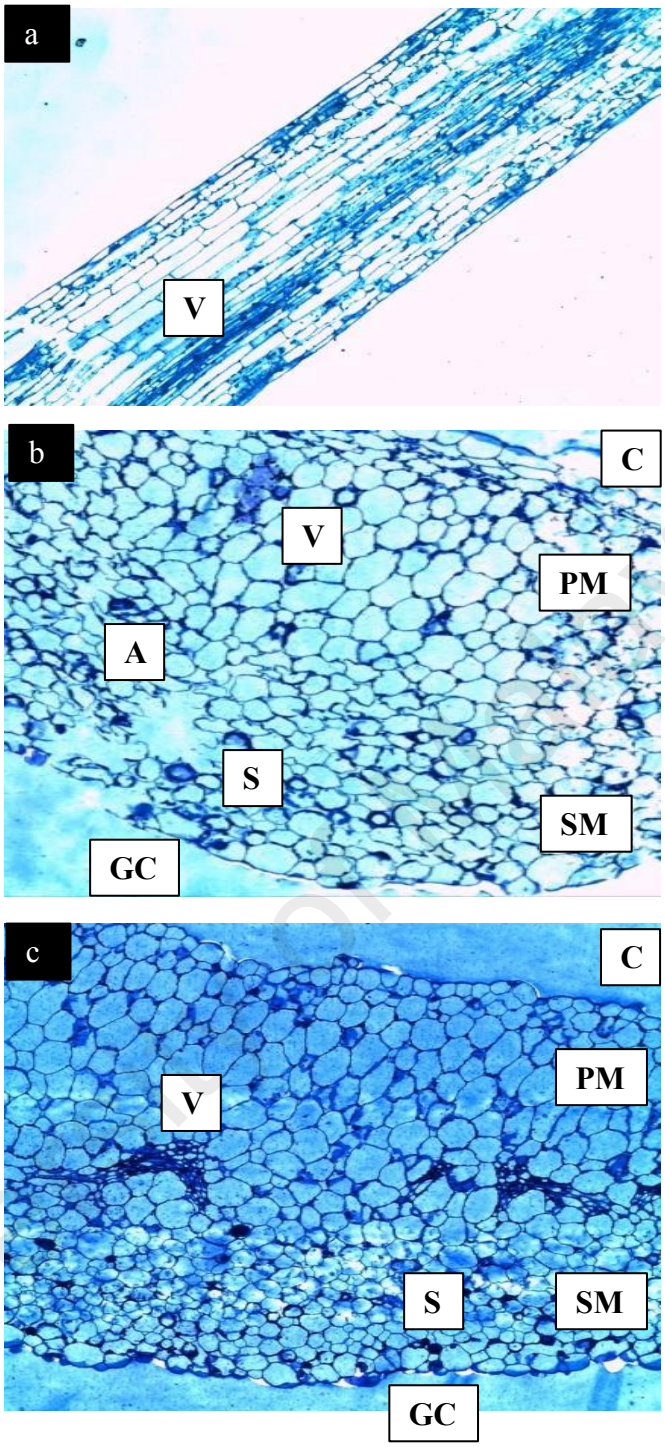


Figure 7.10: Light micrographs from leaves of *Onobrychis viciifolia* Scop. (5 months old) showing morphology and anatomy of cuticle (C) , palisade mesophyll (PM), spongy mesophyll (SM), vascular bundle (V), stoma (S), guard cell (GC) and air space (A), viewed at 40x magnification. (a) Longitudinal section of *in vivo* grown plant. (b) Cross section of *in vivo* plant. (c) Cross section of *in vitro* plant.

7.4 SUMMARY OF RESULTS

1. The distribution of non-glandular trichomes and stomata from *in vivo* and *in vitro* *C. ternatea* L. and *O. viciifolia* Scop. were scattered over the leaf surfaces (abaxial and adaxial).

2. Most of non-glandular trichomes on the *in vitro* leaves for both species were appeared creased and wrinkled.

3. It can be suggested that there were little differences in histological, anatomical and ultrastructural characters between *in vivo* and *in vitro* grown plants of *C. ternatea* L. and *O. viciifolia* Scop., especially in the textures and sizes of the leaves (*in vitro* leaves were smaller compared to *in vivo* grown plants) based on FESEM and histology analysis.

4. Histologically, the adaxial (upper epidermis) from *in vivo* grown leaf of both species showed the presence of cuticle which affected by the sunlight exposure, besides providing protection against desiccation and pathogens. Overall, the ultrastuctural features of *in vivo* leaf showed more air space in palisade and spongy mesophyll layer, while the *in vitro* leaf with compact cells.

5. After 6 months of acclimatization in the green house, the regenerants showed similar growth characteristics as mother plants, implying that no somaclonal variation occurred during *in vitro* growth of this species.

CHAPTER 8

ANTIMICROBIAL PROPERTIES OF *Clitoria ternatea* L. AND *Onobrychis viciifolia* Scop. AGAINST BACTERIA (*Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli)* AND FUNGI (*Fusarium* sp., *Candida albicans* AND *Trichoderma* sp.)

8.1 EXPERIMENTAL AIMS

Recently, tissue culture study also focused on comparison of antimicrobial activities from *in vivo* (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. Under sterile condition, *in vitro* callus or regenerants may contribute natural sources for pharmaceuticals, food supplement, cosmetics and safety colorant production. The United State Development Agency (2014) intends to conserve *Clitoria ternatea* L. along with the other 16 leguminous species with potentially beneficial for phytochemicals. Worldwide known this species as medicinal plant that possess pharmacological activities including antimicrobial, anti-oxidant, anti inflamatory, antipyretic, anti-helminthic and analgesic activities (Mukherjee et al., 2008; Gupta et al., 1968). Plant extracts containing bioactive agents with antimicrobial properties that have been found useful in treating bacterial and fungal infections (Adeniyi et al., 2010), as well as responded as multiple antibiotic resistance (Kuete et al., 2012).

Antimicrobial activity has been focused increasingly due to the demands of antibiotic. According to Guo et al. (2004), ethanol was the best extract solvent. Chloramphenicol (as positive control), is an antibiotic that naturally produced by *Streptomyces*. Thus, to fulfil the increasing demand of this potent medicinal plant, *in vitro* culture is an alternative method for sterile mass propagation.

The specific aims of this chapter were:

1. To investigate the antibacterial activity from *in vivo* leaves, *in vitro* leaves and callus of ethanolic extracts of *C. ternatea* L. and *O. viciifolia* Scop. against *Bacillus subtilis, Staphylococcus aureus and Escherichia coli.*

2. To investigate the antifungal activity from *in vivo* leaves, *in vitro* leaves and callus of ethanolic extracts of *C. ternatea* L. and *O. viciifolia* Scop. against *Fusarium* sp., *Candida albican* and *Trichoderma* sp.

8.2 MATERIALS AND METHODS

8.2.1 Plant Materials

Based on the results in Chapter 4, the optimum somatic embryogenic callus for regeneration were obtained from root explants of *C. ternatea* L.(0.43±0.02 g on MS+40.0 mg/L $ADSO_4$+2.0 mg/L KIN) and *O. viciifolia* Scop. (1.05±0.01g on MS+40.0 mg/L $ADSO_4$+1.0 mg/L NAA+2.0 mg/L BAP). The cultures were incubated and maintained at 25±1°C with 16 hours light and 8 hours dark. The samples (4-month-old *in vivo* grown leaves, 4-month-old *in vitro* grown leaves and 2-month-old *in vitro* callus) were sealed in aluminium foil and freeze dried (72 hours). Then, the dried samples were blender into powder form and kept in -20±1°C freezer.

8.2.2 Preparation of Ethanolic Extraction

The method for ethanolic extraction followed Rahiman et al. (2013) with minor modification. The freeze-dried samples (0.2 g) were soaked in 10 ml alcohol for 24 hours at room temperature under dark condition to avoid degradation and oxidixation (Figure 8.1). The mixtures were then centrifuged at 5000 rpm for 10 min. Subsequently, rotary evaporator was used to remove alcohol content in the solution by evaporation under reduced pressure at relative low temperature (<30ºC).

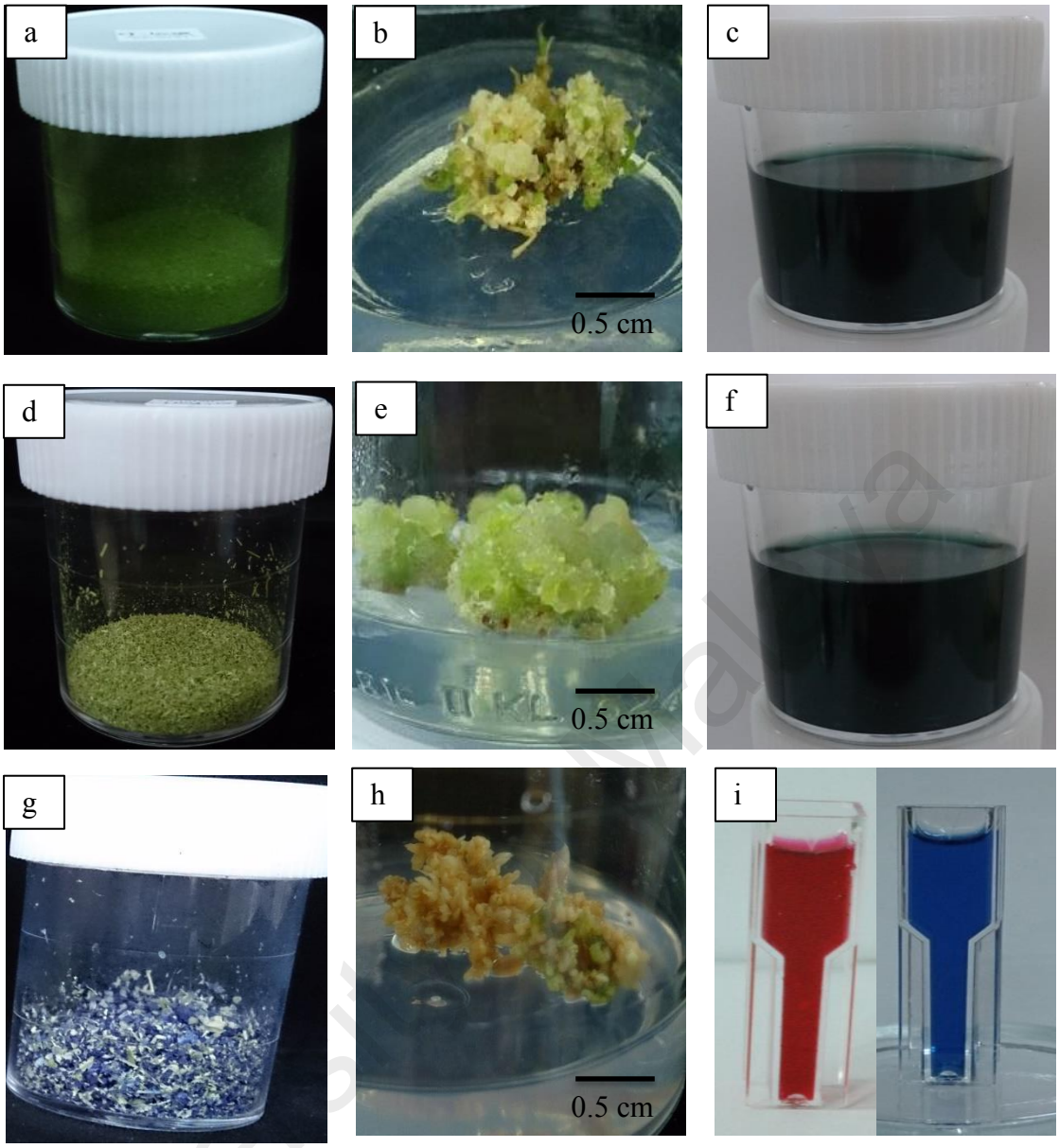


Figure 8.1 : Ethanolic extraction from leaves, callus and flowers of Clitoria ternatea L. and *Onobrychis viciifolia* Scop. (a) Freeze dried leaves of *Clitoria ternatea* L. (b) Callus from root explant of *Clitoria ternatea* L. cultured on MS+40.0 mg/L $ADSO_4$+2.0 mg/L KIN. (c) Green samples extraction. (d) Freeze-dried leaves of *Onobrychis viciifolia* Scop. (e) Callus from root explant of *Onobrychis viciifolia* Scop. cultured on MS+40.0 mg/L $ADSO_4$+1.0 mg/L 2,4-D+3.0 mg/L KIN. (f) Green extraction of *Onobrychis viciifolia* Scop. (g) Freeze-dried petals of *Clitoria ternatea* L. from root explant. (h) Callus from flower bud of *Clitoria ternatea* L. (i) Red ethanolic extract and blue aqueous extract from *Clitoria ternatea* L. petals.

8.2.3 Paper Disc Diffusion Technique

The antimicrobial properties were determined by using agar diffusion technique using paper disc (Bauer et al., 1966). Three bacteria (*Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*) and fungi (*Fusarium* sp., *Aspergillus niger* and *Trichoderma* sp.) were streaked on bacteria agar (BA) and dextrose agar (PDA), respectively, using hockey steak. Subsequently, the sterilled paper discs (6 mm diameter) were pipetted with 20 ul of chloramphenicol (as control for antibacterial), carbendazim (as control for antifungal), *in vivo* extracts and *in vitro* extracts. The controls were in 0.1 mg/L 10^{10} CFU/ml dissolved in ethanol. The plates were incubated at room temperature for 48 hours and the inhibition zones were measured. Antimicrobial agents were recognized by the formation of inhibition zones that kills or inhibits the growth of microorganisms (Jagessar et al., 2009). Three replicates were used for each microorganism tested.

8.2.4 Data Analysis

All experiments were conducted using a completely randomized design. Data collected were statistical analyzed using Duncan's Multiple Range Test (DMRT). Mean with different letters in the same column differ significantly at $p<0.05$.

8.3 RESULTS

8.3.1 Antibacterial Activity of *Clitoria ternatea* L. against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*

8.3.1.1 Antibacterial Activity of *Clitoria ternatea* L. from Leaves and Callus Extracts, Grown *In Vivo* and *In Vitro*

Table 8.1 and Figure 8.2 showed the antibacterial activity of ethanolic extracts from *in vivo* leaves, *in vitro* leaves and callus of *C. ternatea* L. against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*. The highest inhibition zone (16.00 mm) obtained from *in vivo* leaves against both *Bacillus subtilis* and *Escherichia coli*. This followed by the different inhibition zone (15 mm) from *in vitro* leaves that only showed activity against *Bacillus subtilis* and callus (15 mm) against *Escherichia coli*, only. The lowest inhibition zones (11 mm) were obtained from ethanolic extracts from callus and *in vitro* leaves, against *Bacillus subtilis* and *Escherichia coli*, respectively. These results contradict with chloramphenicol (control) that successfully inhibited the growth of *Staphylococcus aureus* (33.00±0.67 mm). Generally, when compared to the mother plant of *C.ternatea* L. (*in vivo* leaf) that showed the best inhibition zone for the growth of both *Bacillus subtilis* and *Escherichia coli*, *in vitro* leaves (the best against *Bacillus subtilis*) and callus (the best against *Escherichia coli*) also showed the same activity.

Table 8.1 : Antibacterial activity of ethanolic extracts of *Clitoria ternatea* L. against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*.

Bacteria (10^{10} CFU/ml)	Mean inhibition zone diameter, mm			
	Chloramphenicol (0.1 g/100 ml)	*In vivo* leaf	*In vitro* leaf	Callus
Bacillus subtilis	**23.00±0.59a**	16.00±0.35b	15.00±0.56b	11.00±0.46c
Staphylococcus aureus	**33.00±0.67a**	13.00±0.55b	13.00±0.57b	12.00±0.41b
Escherichia coli	**22.00±0.58a**	16.00±0.47b	11.00±0.52c	15.00±0.37b

Values are Mean±standard error included the diameter of filter paper disc (6.00 mm). The bold numbers represent the optimum result. n=3.

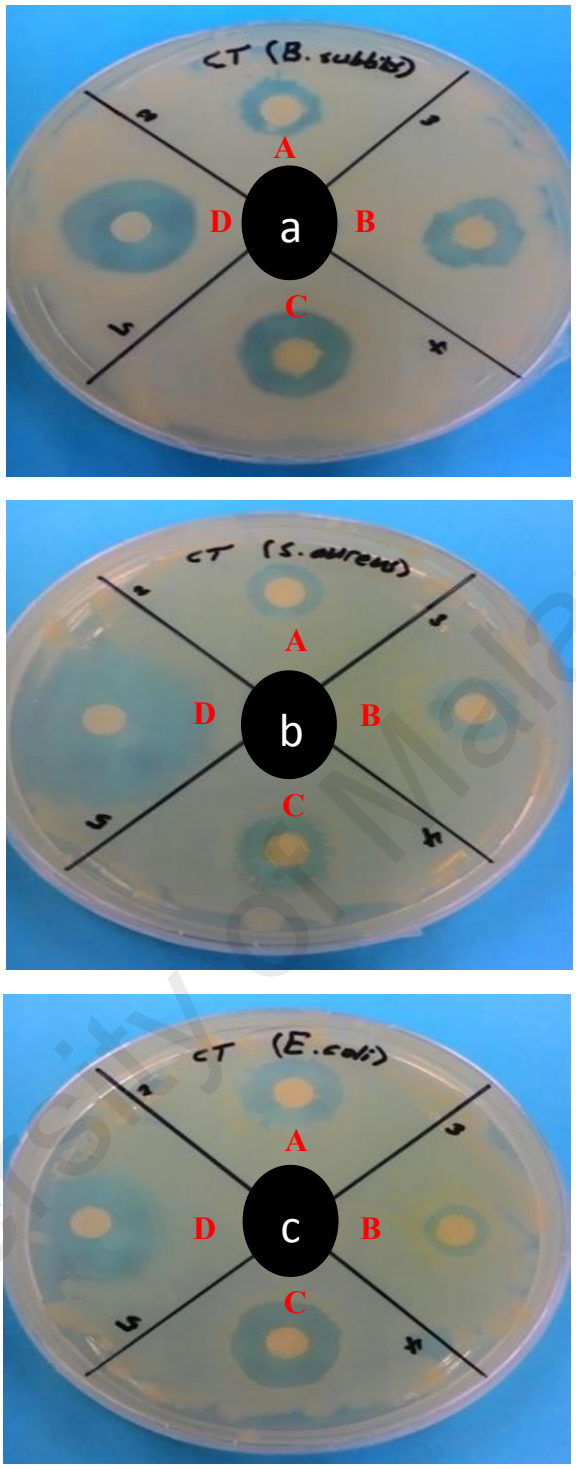


Figure 8.2 : Antibacterial activity of *Clitoria ternatea* L. using diffusion disc technique from ethanolic extracts of callus (A), *in vitro* leaves (B), *in vivo* leaves (C) against bacteria. (a) *Bacillus subtilis.* (b) *Staphylococcus aureus.* (c) *Escherichia coli.;* Chloramphenicol as control (D). Callus obtained from root explants.

8.3.1.2 Antibacterial Activity of *Clitoria ternatea* L. from Flower and Callus Extracts, Grown *In Vivo* and *In Vitro*

For antibacterial activity (Table 8.2 and Figure 8.3), both *in vivo* and *in vitro* ethanolic extracts from vivid blue flowers of *C. ternatea* L. showed the highest antibacterial activity against the same bacteria (*Bacillus subtilis*) with 11 mm and 10 mm inhibition zones, respectively. Furthermore, the lowest antibacterial activity against the same bacteria (*Escherichia coli* with the same inhibition zone (8.0 mm). However, *Staphylococcus aureus* responded differently with higher inhibition zone from *in vitro* ethanolic callus extract (10.0 mm) compared to *in vivo* extract (7.0 mm). The highest inhibition zone of chloramphenicol (control) resulted against *Staphylococcus aureus* with 28 mm inhibition zone, followed by *Escherichia coli* (20.0±0.3 mm) and *Bacillus subtilis* (17.0±0.3 mm). No inhibition zone obtained from *in vivo* aqueous extract.

Table 8.2 : Antibacterial activity of ethanolic and aqueous flower extracts of *Clitoria ternatea* L. against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*.

Bacteria (10^{10} CFU/ml)	Mean inhibition zone diameter, mm			
	Chloramphenicol (0.1 g/100 ml)	*In vitro* ethanolic callus	*In vivo* ethanolic flower	*In vivo* aqueous flower
Bacillus subtilis	**17.0±0.3a**	11.0±0.3b	10.0±0.3b	0
Staphylococcus aureus	**28.0±0.6a**	10.0±0.0b	10.0±0.0b	0
Escherichia coli	**20.0±0.3a**	8.0±0.6b	8.0±0.5b	0

Values are Mean ± Standard Error (SE) included the diameter of filter paper disc (6.00 mm). The bold numbers represent the optimum result. n=3. *In vitro* callus sample was derived from flower explant.

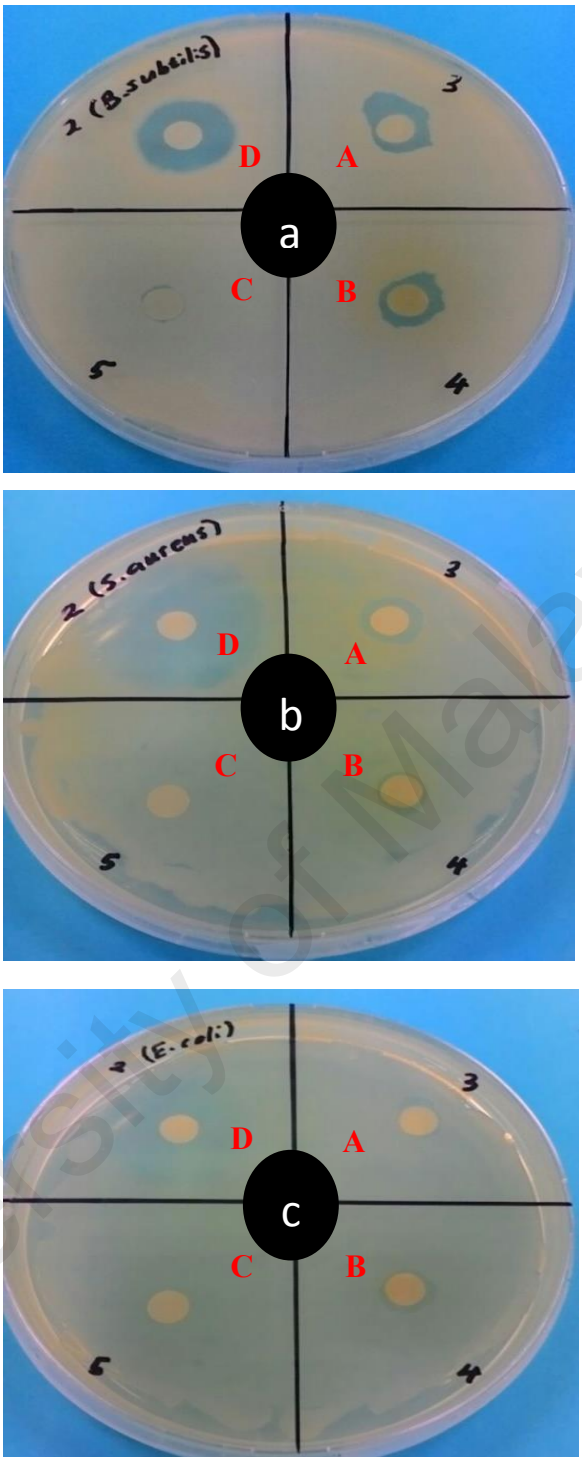


Figure 8.3 : Antibacterial activity of *Clitoria ternatea* L. using diffusion disc technique from ethanolic extracts of callus (A), ethanolic extract of flower (B) and aqueous extract of flower (C) against bacteria. (a) *Bacillus subtilis.* (b) *Staphylococcus aureus.* (c) *Escherichia coli.;* chloramphenicol as control (D), Callus obtained from flower bud explants.

8.3.2 Antibacterial Activity of *Onobrychis viciifolia* Scop. against *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli*

Table 8.3 and Figure 8.4 showed antibacterial activity of ethanolic extracts of *O. viciifolia* Scop. against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*. The highest inhibition zone (17.00±0.65 mm) was obtained from *in vivo* leaves ethanolic extract against *Escherichia coli*, followed by callus extract (15.00±0.47mm) against *Staphylococcus aureus*, which was similar to chloramphenicol (control). Similar to antibacterial activity by *C. ternatea* L. extractions (Table 8.1), the lowest inhibition zones (11 mm) were obtained from ethanolic extracts of *in vitro* leaves and callus, against *Bacillus subtilis* and *Escherichia coli*, respectively (the other way around as compared to results in *C. ternatea* L.).

Generally, the inhibition activity against *Bacillus subtilis* for all samples extractions (*in vivo* leaf, *in vitro* leaf and callus) of *O. viciifolia* Scop.was not as efficient as compared to the leaves from the mother plant. However, the ability of callus extract was comparable to mother plants against *Staphylococcus aureus*. Thus, both *in vitro* leaf and callus extracts have high potentials as anti-*Staphylococcus aureus*.

Table 8.3 : Antibacterial activity of ethanolic extracts of *Onobrychis viciifolia* Scop. against *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli*.

Bacteria (10^{10} CFU/ml)	Mean inhibition zone diameter, mm			
	Chloramphenicol (0.1 g/100 ml)	*In vivo* leaf	*In vitro* leaf	Callus
Bacillus subtilis	**23.00±0.59a**	11.00±0.52b	11.00±0.51b	13.00±0.56b
Staphylococcus aureus	**33.00±0.67a**	16.00±0.61b	12.00±0.39c	15.00±0.47b
Escherichia coli	**22.00±0.58a**	17.00±0.63b	12±0.57c	11.00±0.68c

Values are Mean±standard error included diameter of filter paper disc (6.00 mm). The bold numbers represent the optimum result. n=3. NR=no response.

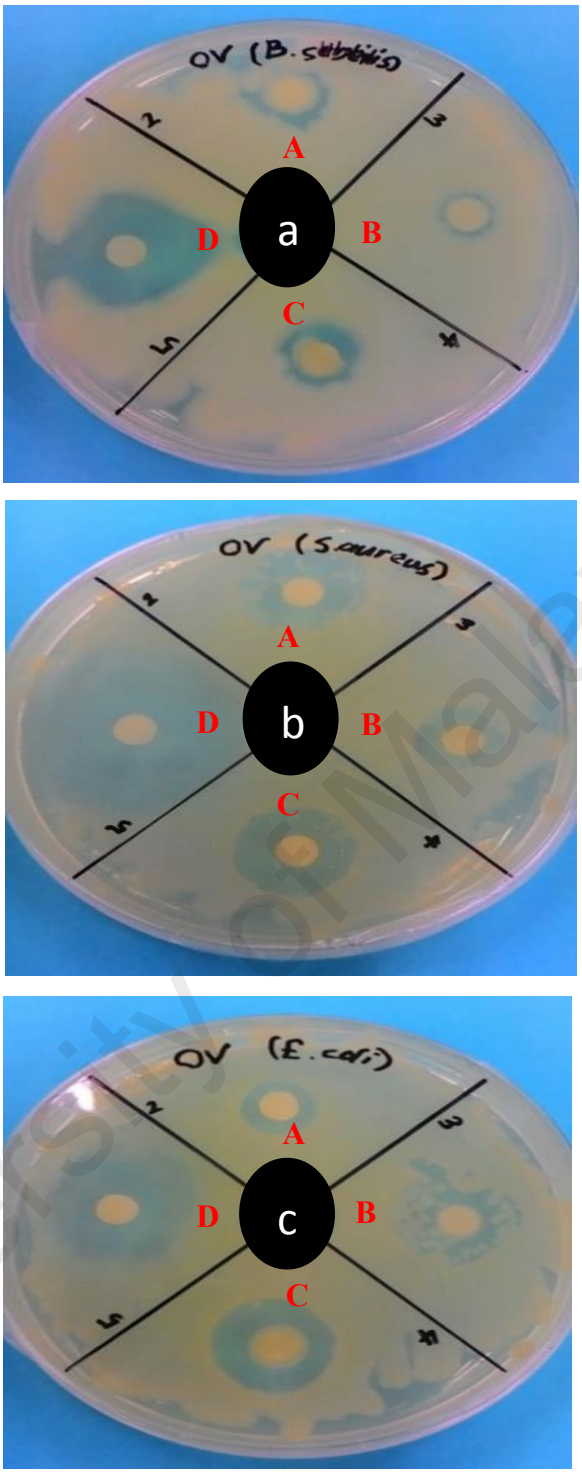


Figure 8.4 : Antibacterial activity of *Onobrychis viciifolia* Scop. using diffusion disc technique from ethanolic extract of callus (A), *in vitro* leaves (B) and *in vivo* leaves (C) against bacteria. (a) *Bacillus subtilis. (*b) *Staphylococcus aureus.* (c) *Escherichia coli.;* chloramphenicol as control (D). Callus was obtained from root explants.

8.3.3 Antifungal Activity of *Clitoria ternatea* L against *Fusarium sp*., *Candida albicans* and *Trichoderma sp*.

8.3.3.1 Antifungal Activity of *Clitoria ternatea* L. from Leaves and Callus Extracts, Grown *In Vivo* and *In Vitro*

Table 8.4 and Figure 8.5 showed the antifungal activity of ethanolic extracts from *in vivo* leaves, *in vitro* leaves and callus of *C. ternatea* L. against *Fusarium sp*., *Candida albicans*, *Trichoderma sp*. The highest inhibition zone (15.00±0.55 mm) was obtained from *in vivo* leaves extract against *Candida albicans*. This followed by callus extraction (14.00±0.46 mm) against *Fusarium* sp. While, *in vitro* leaves extraction were against both *Fusarium* sp. (13.00±0.32 mm) and *Candida albicans* (13.00±0.57 mm). The same lowest inhibition zones (11.00 mm) was obtained from *in vivo* leaves extract against *Fusarium* sp, which contradict to callus extract against *Candida albican*. Unfortunately, no antifungal activity for *Trichoderma* sp. was obtained. Carbendazim (as control) inhibited against *Fusarium* sp. (33.00±0.59 mm), similar to callus extract. Generally, when compared to mother plant, *in vitro* leaves extract had the same ability to inhibit *Candida albicans* growth, while callus extraction showed more efficient inhibition against *Fusarium* sp.

Table 8.4 : Antifungal activity of ethanolic extracts of *Clitoria ternatea* L. against *Fusarium sp*., *Candida albicans* and *Trichoderma sp*.

Fungi (10^{10} CFU/ml)	Mean inhibition zone diameter, mm			
	Carbendazim (0.1 g/100 ml)	*In vivo* leaf	*In vitro* leaf	Callus
Fusarium sp.	**33.00±0.59a**	11.00±0.35c	13.00±0.32b	14.00±0.46b
Candida albicans	12.00±0.67b	**15.00±0.55a**	13.00±0.57b	11.00±0.41c
Trichoderma sp.	NR	NR	NR	NR

Values are Mean±standard error included diameter of filter paper disc (6.00 mm). The bold numbers represent the optimum result. n=3. NR=no response.

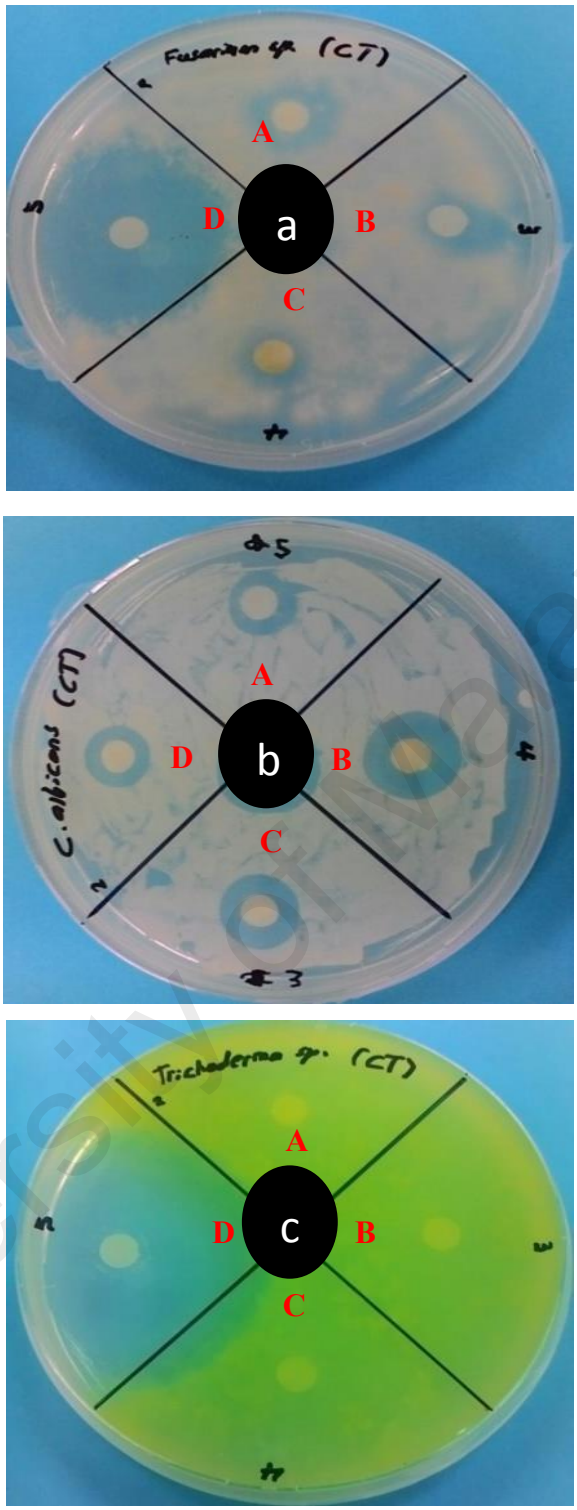


Figure 8.5 : Antifungal activity of *Clitoria ternatea* L. using diffusion disc technique from ethanolic extracts of callus (A), *in vitro* leaves (B) *and in vivo* leaves (C) against fungi. (a) *Fusarium sp*. (b) *Candida albicans* (c) *Trichoderma sp*, ; carbendazim as control (D), Callus obtained from root explants.

8.3.3.2 Antifungal Activity of *Clitoria ternatea* L. from Flower and Callus Extracts, Grown *In Vivo* and *In Vitro*

Uniquely, in this experiment, all samples respond differently against all tested fungi. Antifungal activity (Table 8.5 and Figure 8.6) of *in vitro* ethanolic callus (which derived from flower explant) extract was observed against *Trichoderma* sp. (12 mm), followed by *Candida albican* (10.0±0.2 mm) and *Fusarium* sp. (9.0±0.7 mm). Contrary to *in vivo* ethanolic extract which was against *Fusarium* sp. (10 mm), followed by *Trichoderma* sp. (8.0±0.5 mm). Carbendazim inhibited the growth of *Trichoderma* sp. (28 mm),which was similar to respond obtained from *in vitro* ethanolic callus extract, followed by *Fusarium* sp. (20.0±0.4 mm) and *Candida albican* (17.0±0.6 mm). No inhibition zone observed from *in vivo* aqueous flower extract.

Table 8.5 : Antifungal activity of ethanolic and aqueous flower extracts of *Clitoria ternatea* L. against *Fusarium* sp., *Candida albicans*, *Trichoderma* sp.

Fungi (10^{10} CFU/ml)	Mean inhibition zone diameter, mm			
	Carbendazim (0.1 g/100 ml)	*In vitro* ethanolic callus	*In vivo* ethanolic flower	*In vivo* aqueous flower
Fusarium sp.	**20.0±0.4a**	9.0±0.7b	10.0±0.6b	NR
Candida albicans	**17.0±0.6a**	10.0±0.2b	NR	NR
Trichoderma sp.	**28.0±0.3a**	12.0±0.3b	8.0±0.5c	NR

Values are Mean±standard error included diameter of filter paper disc (6.00 mm). The bold numbers represent the optimum result. n=3. NR=no response.

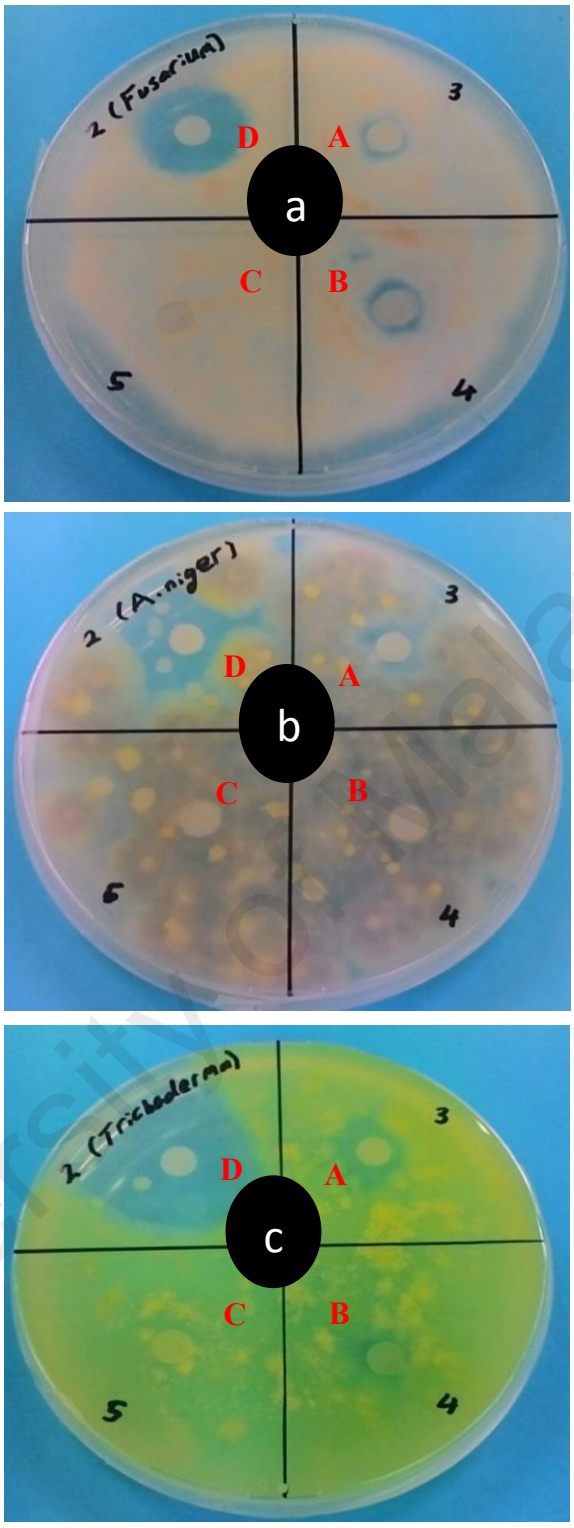


Figure 8.6 : Antifungal activity of *Clitoria ternatea* L. using diffusion disc technique from ethanolic extracts of callus (A), ethanolic extract of flower (B) and aqueous extract of flower (C) against fungi. (a) *Fusarium sp*. (b) *Candida albicans.* (c) *Trichoderma sp,* ; carbendazim as control (D), Callus obtained from flower bud explants.

8.3.4 Antifungal Activity of *Onobrychis viciifolia* Scop. against *Fusarium* sp., *Candida albicans* and *Trichoderma* sp., Grown *In Vivo* and *In Vitro*

Table 8.6 and Figure 8.7 showed the antifungal activity of ethanolic extracts from *in vivo* leaves, *in vitro* leaves and callus of *O. viciifolia* Scop. against *Fusarium sp.*, *Candida albicans* and *Trichoderma sp.* The highest inhibition zone (18 mm) was observed from *in vivo* leaf against both *Fusarium* sp. and *Candida albicans*, subsequently, followed by callus (15-16 mm) and *in vitro* leaves (11.00 mm). Callus extract gave to *Fusarium* sp. **(**16.00±0.46 mm) and *Candida albican* (15.00±0.41 mm).

Among the samples, callus (16.00±0.46 mm) was detected comparable to inhibit the growth *Fusarium* sp., besides in vivo leaf (18.00±0.35 mm). It revealed that *O. viciifolia* Scop. can be served as double functions for anti-*Fusarium* sp. and anti-*Candida albicans*. This result was contrary to carbendazim (as control) that inhibited better against *Fusarium* sp (35.00±0.59 mm) when compared to *Candida albicans* (11.00±0.67 mm). All samples gave no response to *Trichoderma* sp.

Table 8.6 : Antifungal activity of ethanolic extracts of *Onobrychis viciifolia* Scop. against *Fusarium* sp., *Candida albicans* and *Trichoderma* sp.

Fungi (10^{10} CFU/ml)	Mean inhibition zone diameter, mm			
	Carbendazim (0.1 g/100 ml)	*In vivo* leaf	*In vitro* leaf	Callus
Fusarium sp.	**35.00±0.59a**	18.00±0.35b	11.00±0.56c	16.00±0.46b
Candida albicans	11.00±0.67c	**18.00±0.55a**	11.00±0.57c	15.00±0.41b
Trichoderma sp.	NR	NR	NR	NR

Values are Mean±standard error included diameter of filter paper disc (6.00 mm). The bold numbers represent the optimum result. n=3. NR=no response.

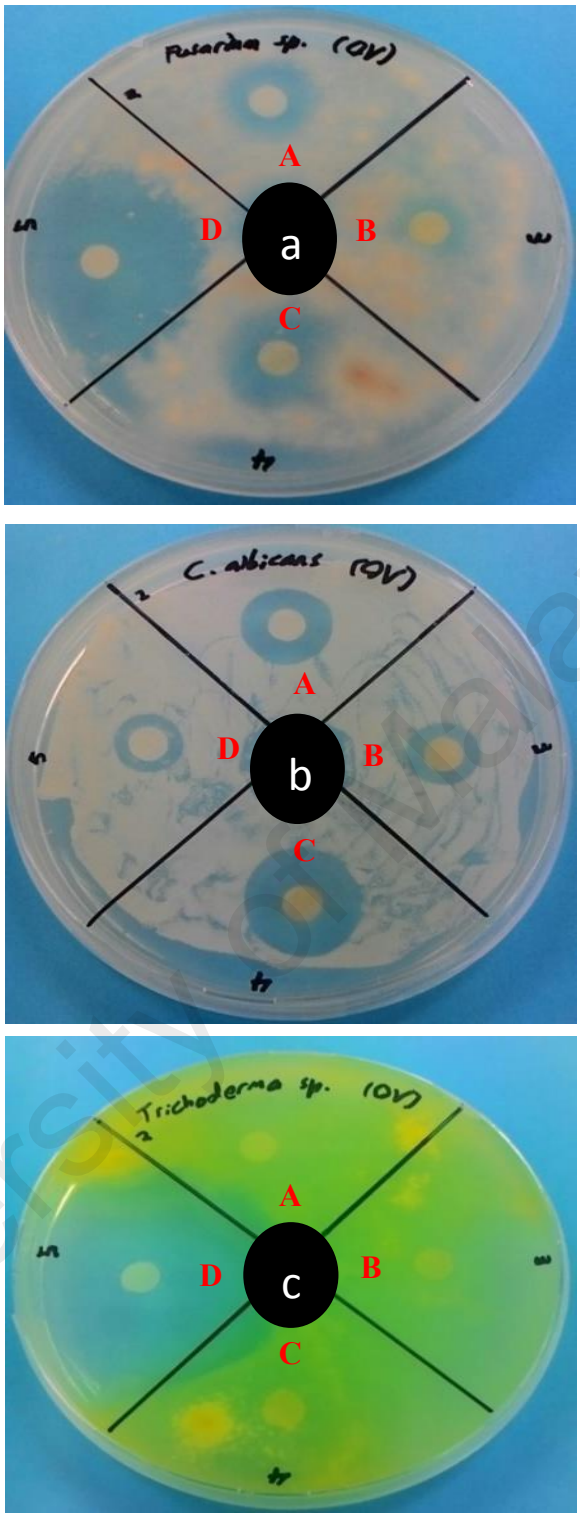


Figure 8.7 : Antifungal activity of *Onobrychis viciifolia* Scop. using diffusion disc technique from ethanolic extracts of callus (A), *in vitro* leaves (B) *and in vivo* leaves (C) against fungi. (a) *Fusarium sp*. (b) *Candida albicans* . (c) *Trichoderma sp,* ; carbendazim as control (D), Callus obtained from root explants.

8.4 SUMMARY OF RESULTS

1. The highest inhibition zone (16.00 mm) was obtained from *in vivo* leaves extraction (mother plant) of *C. ternatea* L. against both (double function) *Bacillus subtilis* and *Escherichia coli*. Whilst, *in vitro* leaves extraction can be proposed as anti-*bacillus subtilis* (15.00±0.56 mm), and callus extraction as anti-*Escherichia coli* (15.00±0.37 mm).

2. The highest inhibition zone (17.00±0.65 mm) was observed from *in vivo* leaves extraction of *O. viciifolia* Scop. against *Escherichia coli*, while, the ability of callus extraction (15.00±0.47 mm) was comparable to mother plants (16.00±0.61 mm) against *Staphylococcus aureus*.

3. The highest inhibition zone (15.00±0.55 mm) of antifungal activity of *C. ternatea* L. obtained from *in vivo* leaves extraction against *Candida albicans*, as well as from *in vitro* leaves extraction (13.00±0.57 mm). Among *in vivo* and *in vitro* leaves extraction, callus performed the best in inhibiting *Fusarium* sp. (14.00±0.46 mm).

4. All extractions of *O. viciifolia* Scop. (*in vivo* leaves, *in vitro* leaves and callus), gave positive response as antifungal activity against both *Fusarium* sp. and *Candida albicans*. However, the highest inhibition zone (18 mm) was seen by *in vivo* leaves extraction , followed by callus extraction (15-16 mm) and *in vitro* leaves extraction (11.00 mm).

5. Both species (*C. ternatea* L. and *O. viciifolia* Scop.), responded negatively to antifungal activity against *Trichoderma* sp. However, *O. viciifolia* Scop. was detected as anti-*Fusarium* sp. and anti-*Candida albicans*.

6. Overall, the present results verified that the antimicrobial activity against bacteria (*Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*) and fungi (*Fusarium* sp., *Candida albicans* and *Trichoderma* sp.) varied with different

explant sources (callus and leaves) of *C. ternatea* L. and *O. viciifolia* Scop., grown *in vivo* and *in vitro*.

7. Overall, both *in vivo* and *in vitro* ethanolic extracts from vivid blue flower of *C. ternatea* L. showed the best antibacterial activity which were against the same bacteria (*Bacillus subtilis*), 11 mm and 10 mm inhibition zones, respectively.

8. However, different antifungal activity (from vivid blue flower explant of *C. ternatea* L.) was detected from *in vitro* ethanolic callus extract (12 mm) which was against *Trichoderma* sp., contrary to *in vivo* ethanolic extract (10 mm) which was against *Fusarium* sp.

CHAPTER 9

ANTHOCYANIN EXTRACTION FROM VIVID BLUE COLOURED FLOWERS OF *Clitoria ternatea* L., GROWN *IN VIVO*

9.1 EXPERIMENTAL AIMS

Vivid blue flowers of *Clitoria ternatea* L. contain flavonoid such as quercetin, kaemferol, robinin and clitorin, besides several glycosides including malvidin-3-*β*-glycosides, dephinidin-3-β-glycoside (Srivastava and Pandey, 1977) and dietary anthocyanins (Terahara et al., 1996). Anthocyanins are responsible for the water soluble, vacuolar, pink, red, purple and blue pigments present in coloured plant pigments. According to Cunningham and Gantt (1998), these pigments are important agronomic value in many crops and ornamental plants. However, anthocyanins (Figure 9.1) are unstable and easily degrade and fade whenever expose to the light.

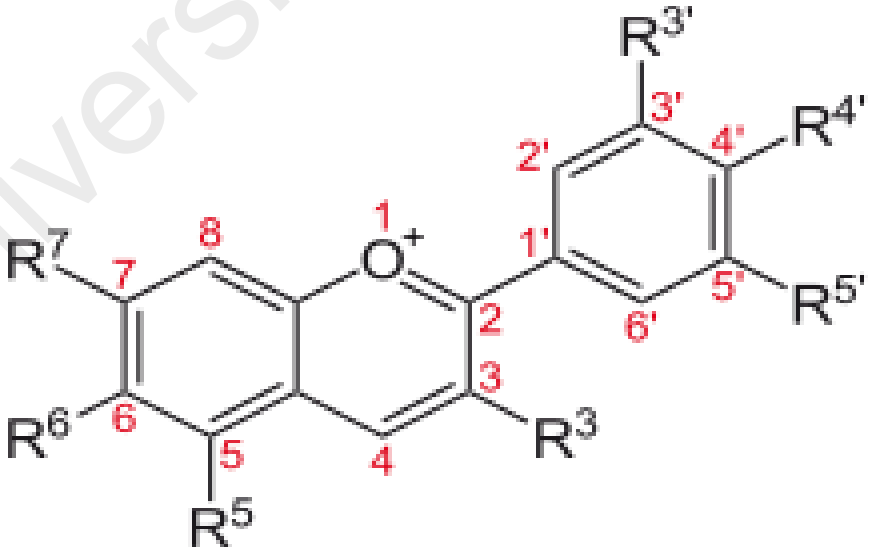


Figure 9.1 : General structure of anthocyanins.

C. ternatea L. is known as medicinal plant and widely used to treat eye, throat, skin and ulcer (Malabadi and Nataraja, 2001), as well as used as phytochemical substances. The phytochemical investigations revealed the presence of saponins, carbohydrates, alkaloids, proteins, anthroquinones and phytosterols. It is used as diuretics, antihelmintic, antidiabetic, antipyretic and brain tonic (Kirtikar and Basu, 1981). The extract of *C. ternatea* L. contains anxiolytic, antidepressant, anticonvulsant and antistress properties (Jain et al., 2003). According to the traditional system of medicine 'Aparajita' is considered as a 'Medhya' drug to improve intelligence and enhance memory function (Kulkani et al., 1988). It is also used in the treatment of chronic bronchitis, dropsy, goiter, leprosy, mucous disorders, sight weakness, skin diseases, sore throat and tumors. In Ayurveda Indian medicine, the roots are most widely used and are bitter, refrigerant, laxative, intellect promoting, diuretic, anthelmintic and as tonic. The root is useful in dementia, burning sensation, inflammation and asthma. The seeds are cathartic, while the leaves are used in otalgia and hepatopathy. In addition, the roots, stems and flowers are recommended for the treatment of snakebite and scorpion-sting. Due to natural colorant safety and health benefit, as well as strong consumer demand for more natural products, *C. ternatea* L. has a great potential to be cultivated on large scale basis in Malaysia with low cost of maintenance, under natural climate with suitable temperature (30±1ºC).

The specific aims of this specific chapter was to investigate some weathering and corrosion test (pH, UV-B radiation, NaCl and heat test) to the stability of anthocyanin from crude extracts of *C. ternatea* L. during storage and would be potentially useful for commercial applications as natural colorants in baby products, cosmetics and coating technology.

9.2 MATERIALS AND METHODS

9.2.1 Plant Materials

The vivid blue flowers of *C. ternatea* L. was collected from the garden of Institute of Biological Sciences, Faculty of Science, University of Malaya. The flowers were sealed in aluminium foil and freeze dried for 72 hours. Then, the dried flowers were blendered into powder form and kept in -20±1°C freezer to preserve the anthocyanin.

9.2.2 Preparation of Anthocyanin Extraction

Dried flower powder (10 g) was dissolved in 100 ml of 0.5% trifluoroacetic acid (TFA) in methanol solution to extract the natural colorant (anthocyanin). The mixtures was then centrifuged at 5000 rpm for 10 min. Subsequently, the supernatant was filtered through Whatman No. 1 filter paper to remove any particles or residues. The filtered supernatant was covered with aluminium foil (dark condition) to avoid degradation and hydrolysis of the acyl groups in the anthocyanin structure. Subsequently, rotary evaporator was used to remove methanol content in the solution by evaporation under reduced pressure at relative low temperature (<30ºC). The pellet was discarded. Subsequently, the extraction was purified with addition (several times) of ethyl acetate and distilled water (1:2:3) to remove stilbenoids, chlorophyll, less polar flavonoids and other non-polar compounds. Finally, the extract was stored under dark condition at 4±1ºC to prevent colour degradation.

9.2.3 Preparation of Stability Tests

9.2.3.1 Effects of pH

The pH of the methanolic extracts were varied from pH 1, 4.5 to 5.5. These acidic samples were changed to different colours according to the pH. The samples were then subjected to UV spectrophotometric analysis in the visible region between

400-700 nm of wavelength and the spectrum profiles were compared. Three replicates for each pH test.

9.2.3.2 Effects of UV Radiation

The samples were exposed to UV lamp which emitted radiation at 312 nm with 17.55 lux irradiation intensity. The distance between samples and UV lamp was fixed at 10 cm. The absorbance measurements were obtained by UV spectrophotometric analysis at 0, 60 and 120 min. Three replicates for each duration were prepared.

9.2.3.3 Effects of Sodium chloride (NaCl) Concentrations

Basically, NaCl was used for salinity test and mimic to the content of seawater. The samples were added with 0, 20 and 30 g/L NaCl. The samples were then subjected to spectrophotometric analysis and the spectrum profiles were compared. Three replicates for each concentration were prepared.

9.2.3.4 Effects of Heating Duration

The anthocyanins were placed in test tube and heated for 10, 20 and 30 min in the oven (50±1ºC). The samples were then subjected to spectrophotometric analysis and the spectrum profiles were compared. Three replicates for each duration were prepared.

9.2.4 Preparation of Coating Materials

9.2.4.1 Preparation of Resin (20% PMMA)

Polymetilmetacrilic (PMMA) powder (20 g) was mixed with solvent (80 ml) tetrahydrofuran (THF) and 20 g acrylicpolyol as binder. THF was heated up and mixed with PMMA and acrylicpolyol until the powder became melted. THF is sensitive to the air. The effectiveness of tartaric acid (added to the colourant-polymer mixture) in stabilising the colour of the coating was also examined.

9.2.4.2 Coating Technique

The pigment-resin solution was coated onto glass slides and dried at a temperature of 30±1°C for 24 hrs. The coatings were added with copigments (1% of tartaric and citric acid) for stability comparison. Determination was done in 3 replicates.

9.2.5 Data Analysis

The spectrum profiles (absorbances) of extractions were measured by UV spectrophotometric analysis in the visible region of 400 to 700 nm (Shimadzu UV-3101PC). All samples were prepared in three replicates.

9.3 RESULTS

9.3.1 Anthocyanin and Stability Properties

Anthocyanin extracted from crude petals of *C. ternatea* L. (Figure 9.2) was easily oxidized when exposed to room temperature. In this experiment, methanol acidified with 0.5% trifluoroacetic acid (TFA) was used for solvent extraction of the anthocyanin, which produced bright red colour solution. The main aim of the test was to observe the effects of weathering including pH (acidity), sunlight exposure (UV rays), sodium chloride (seawater salinity) and heat (temperature) on this natural colorant as potential coating material, as well as to study the cause of corrosion. Generally, the anthocyanin absorbance values decreased with higher pH (Figure 9.2), duration of UV radiation (Figure 9.3), concentrations of sodium chloride (Figure 9.4) and durations of heat (Figure 9.5).

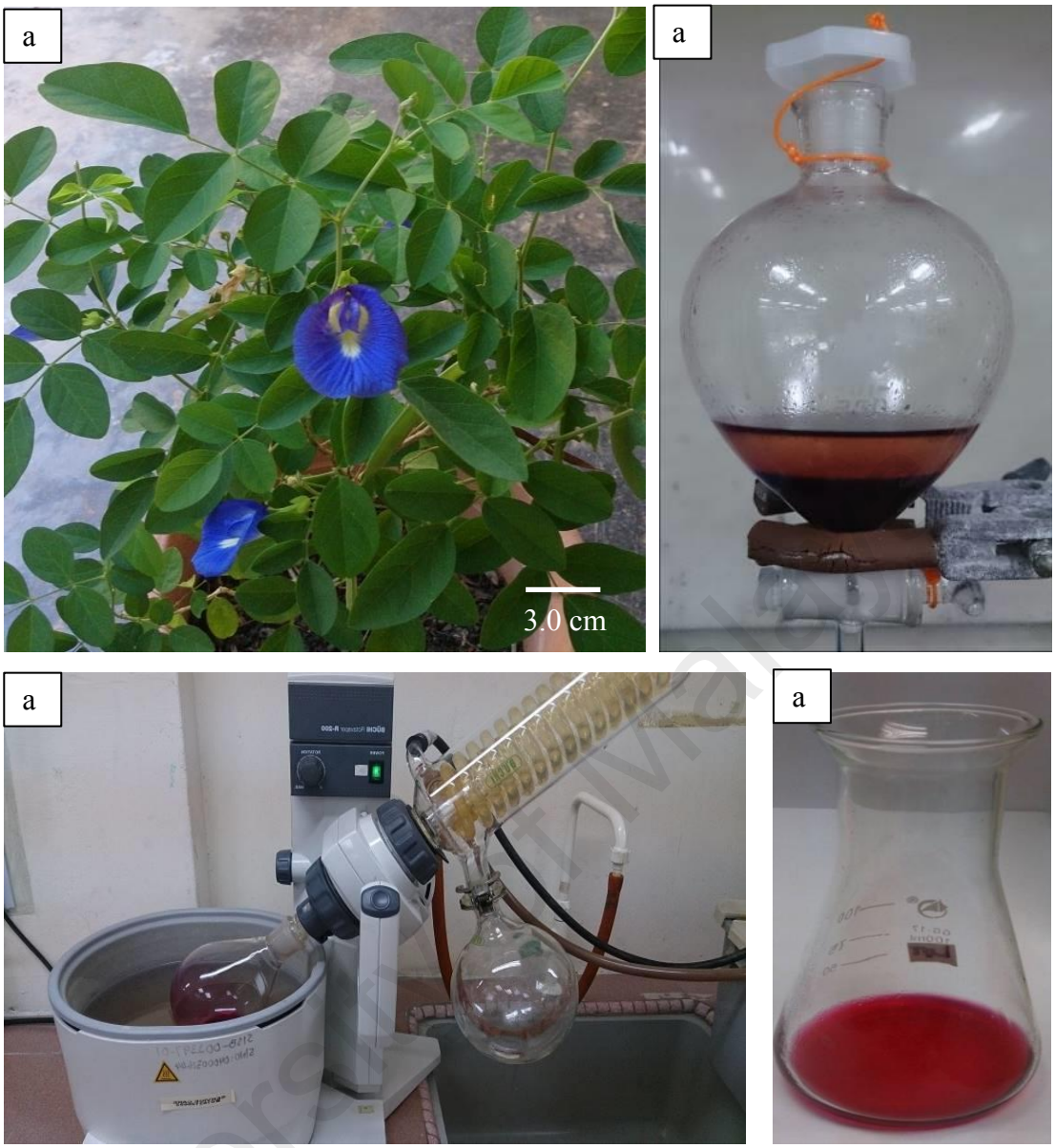


Figure 9.2 : Anthocyanin extraction from *Clitoria ternatea* L. (a) Vivid blue coloured petals. (b) Separation of anthocyanin and ethyl acetate. (c) Rotary evaporator to remove alcohol. (d) Bright red colour at pH 1.

Under normal light condition (fluorescent lamp) at room temperature, the colour of *C. ternatea* L. changed upon the exposure at different pH. The extract from vivid blue coloured of *C. ternatea* L. petals became red at pH 1, purple at pH 4.5, blue at pH 5.5 and cyan at pH 6.5 (Figure 9.3), due to the change in the pigment's structure. This characteristic role as a strong indicator for presence of anthocyanins is the fact that they changed colour with pH as compared to betalain. While, the flower extracted with water showed navy blue colour at pH 5.5. The best

bright red colour extract was observed at pH 1 and pH 4.5. At pH 5.5 and pH 6.5, the red colour changed to green, while at pH 10 and pH 12, the colour changed to brown. The graph in Figure 9.4 showed absorbance values for pH 1, pH 4.5 and pH 5.5. The maximum absorbance (0.6048) was obtained at pH 1 and the minimum (0.4164) at pH 5.5, which was irradiated at 550 nm of wavelength, which was apparent for anthocyanins. Overall, based on the absorption spectra (the fluorescence intensity of the sensor increased with the increase in pH value), the anthocyanins were stable under strongly acidic (pH 1), but unstable in weakly acidic condition (pH 4.5 and 5.5). Therefore, the colour faded faster at higher pH (pH 1) than lower pH (pH 4.5 and 5.5).

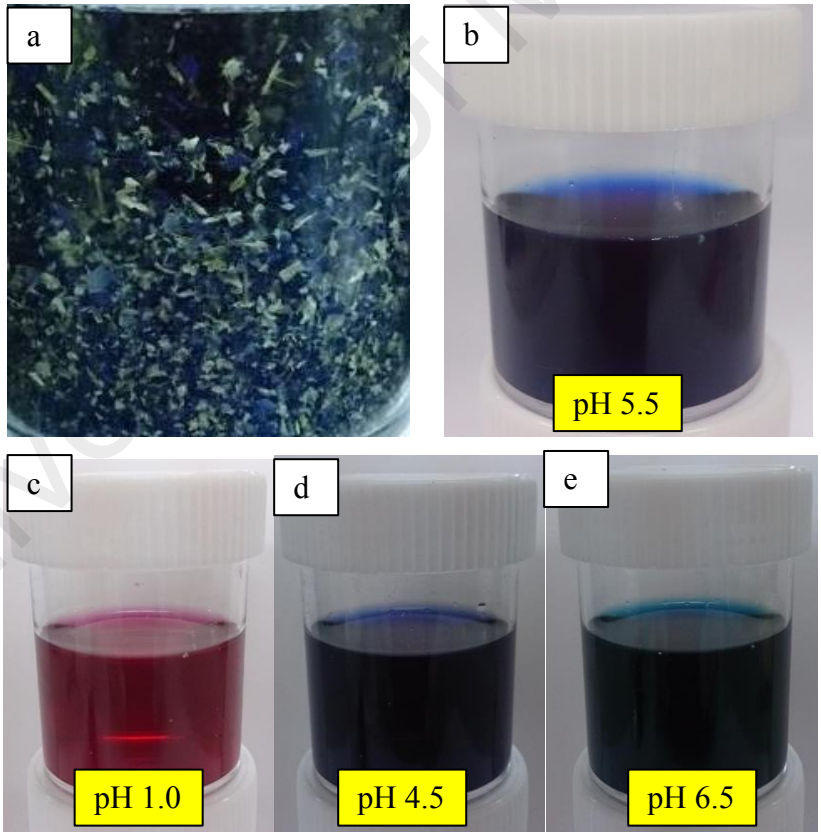


Figure 9.3 : Effects of pH on anthocyanin obtained from *Clitoria ternatea* L. (a) 72 hours freeze-dried petals. (b) Vivid blue aqueous extract at pH 5.5 (c) Red methanolic extract at pH 1. (d) Blue methanolic extract at pH 4.5 (e) Cyan methanolic extract at pH 6.5.

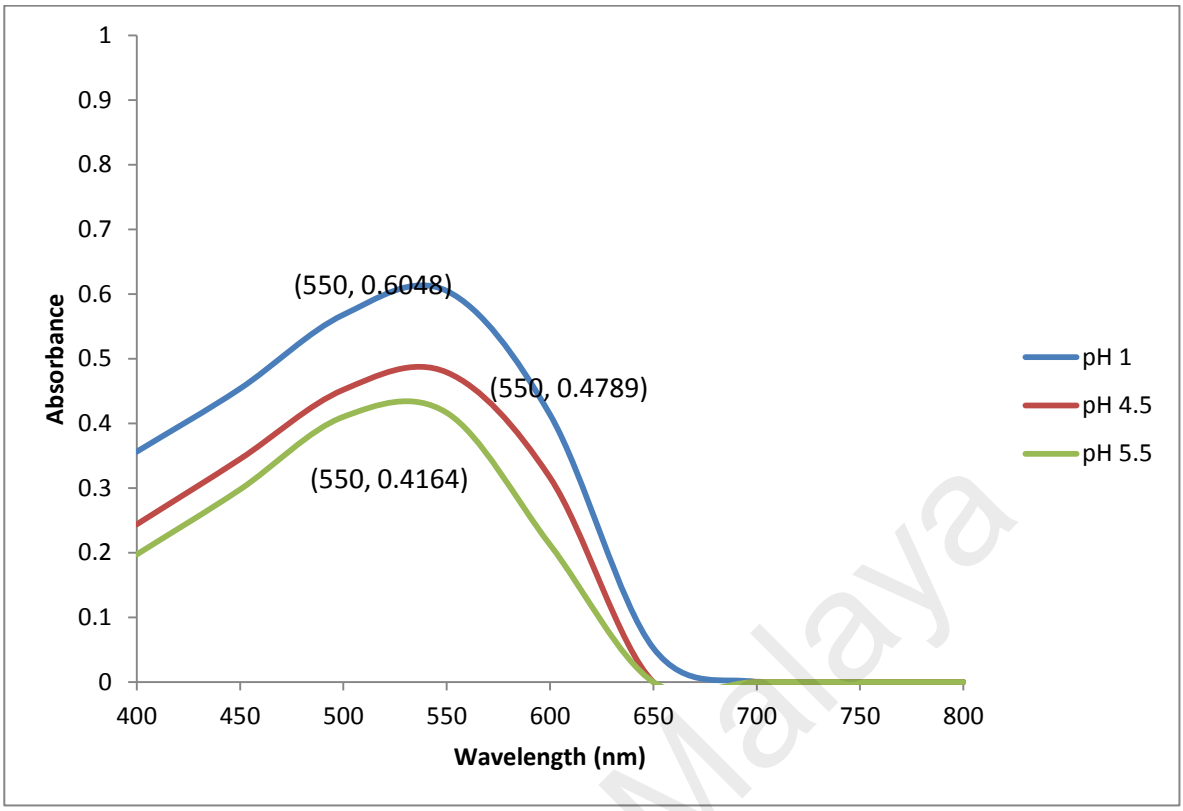


Figure 9.4 : UV-vis spectra of effects of pH (pH 1, 4.5 and 5.5) on absorbance values (irradiated at 400-700 nm) from anthocyanin extraction of vivid blue coloured petals of *Clitoria ternatea* L.

The graph in Figure 9.5 showed absorbance values for UV radiation within 0, 60 and 120 min. Conventionally, UV radiation play an important role in killing microorganisms in food and beverages. In the present study, the results obtained the maximum absorbance (0.6048) in 0 min at 550 nm, whilst the minimum (0.4164) irradiated at 600 nm of wavelength at pH 1. Overall, the absorbance values decreased in higher duration of UV radiation exposure at a faster rate. However the original red colour was remaining the same after 24 hours.

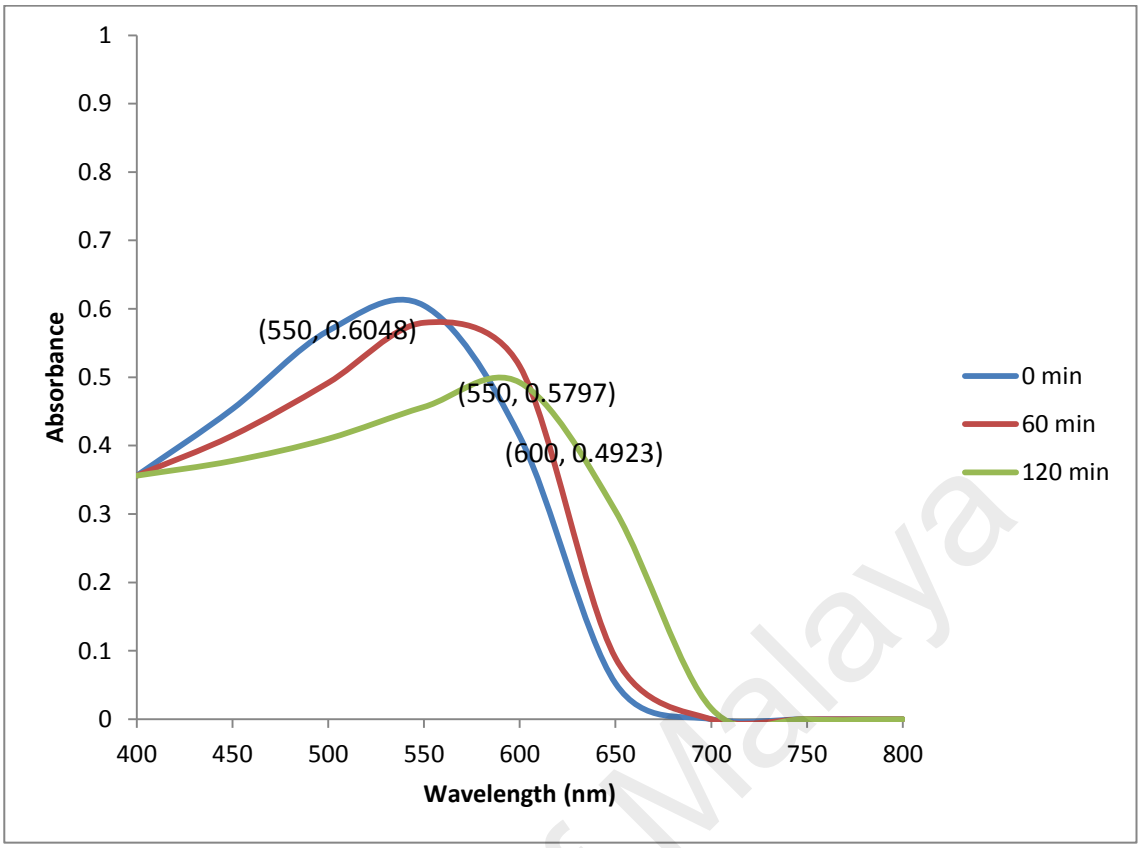


Figure 9.5 : UV-vis spectra of effects of UV radiation (0, 60 and 120 min) on absorbance values (irradiated at 400-700 nm) from anthocyanin extraction of vivid blue coloured petals of *Clitoria ternatea* L.

The graph in Figure 9.6 showed absorbance values for different NaCl concentrations (0, 20 and 30g/L). The results obtained the maximum absorbance (0.6048) in 0 g/L NaCl and minimum (0.4999) in 30 g/L, which was irradiated at 550 nm of wavelength at pH 1. Overall, the absorbance values decreased in higher NaClconcentrations with the same red colour (as original) after 24 hours.

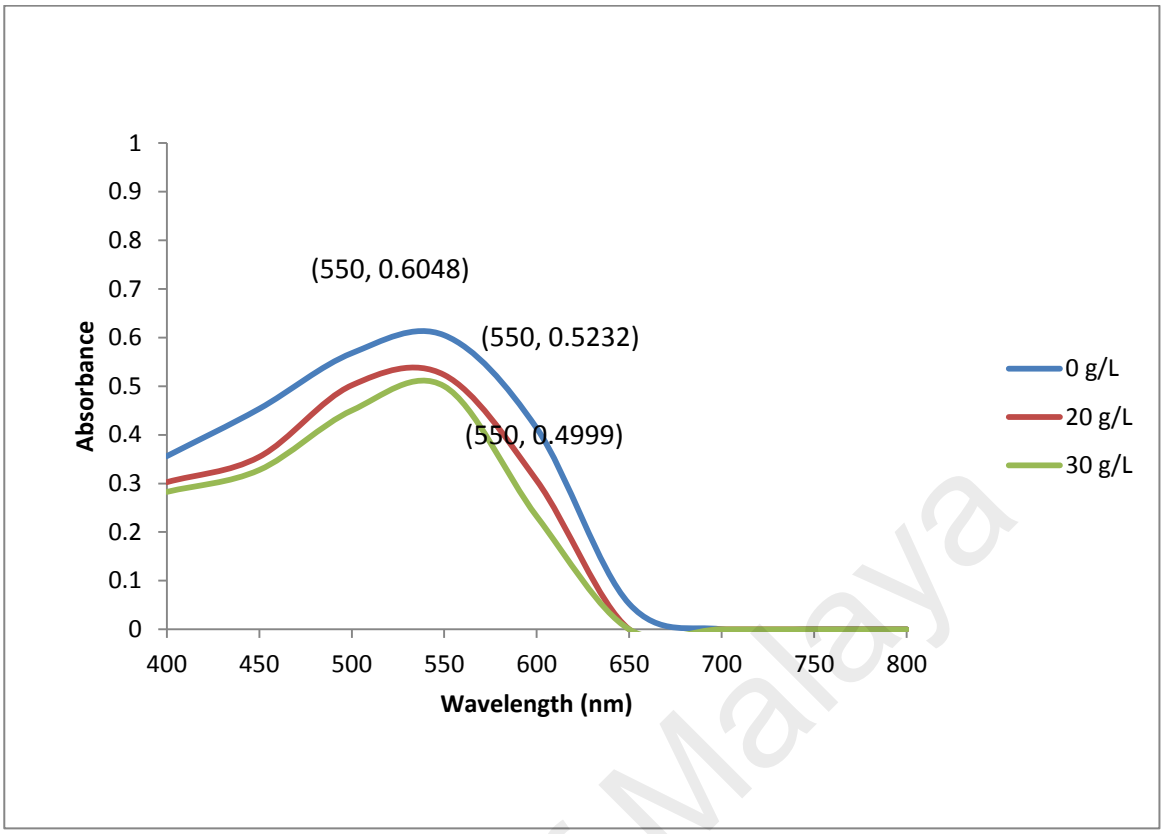


Figure 9.6 : UV-vis spectra of effects of sodium chloride (NaCl) concentrations (0, 20 and 30 g/L) on absorbance values (irradiated at 400-700 nm) from anthocyanin extraction of vivid blue coloured petals of *Clitoria ternatea* L.

Originally, the heating test aims to investigate the effects of hot water or weather to the stability and durability of anthocyanin (natural colorant) such as in production of baby products. During the heating test, the red colour (at pH 1) of this anthocyanin extraction became darker in the oven (50±1ºC) as the duration of heat increased. This characteristic (the colour became darker, instead of fade), which beneficial as a stable natural colourant as shown in Figure 9.7, the absorbance values for different heating duration (0, 20 and 30 min). The results obtained the maximum absorbance (0.6048) in 10 min at 550 nm, whilst the minimum (0.4323) in 30 min irradiated at 600 nm of wavelength at pH 1. Overall, the absorbance values decreased in higher duration of heating in the oven, with the same red colour (as original) after 24 hours.

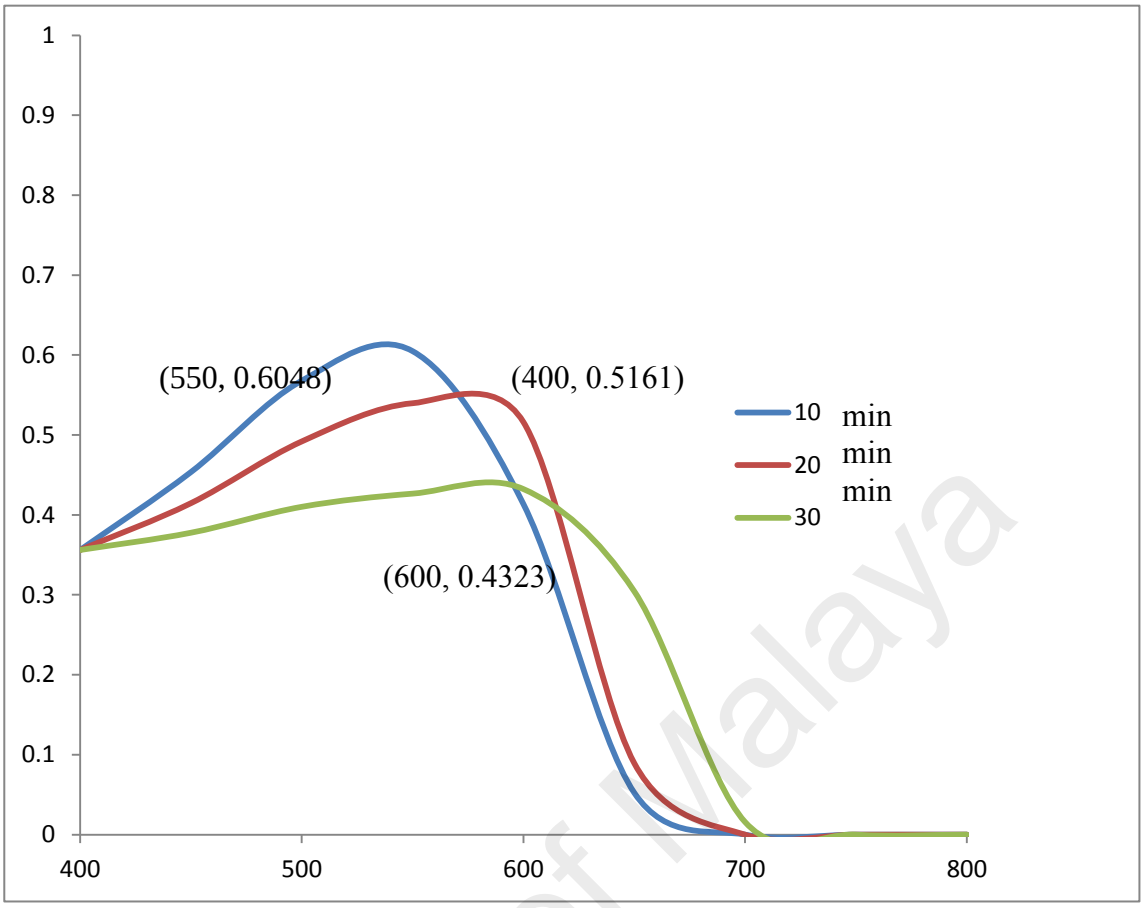


Figure 9.7 : UV-vis spectra of effects of heating at 50±1ºC (0, 20 and 30 min) on absorbance values (irradiated at 400-700 nm) from anthocyanin extraction of vivid blue coloured petals of *Clitoria ternatea* L.

9.3.2 Natural Colourant as Coating Material from *Clitoria ternatea* L.

Anthocyanins extracted from crude petals of *C. ternatea* L. was easily oxidized when exposed to the room temperature. The best bright red colour extract was observed at pH 1. Therefore, in the aim of retaining the bright red color, the anthocyanin extract was mixed with 20% polymethylmetacrylate (PMMA) as co-pigmentation effect of organic acids (tartaric and citric acid) at pH 2 were coated onto glass slides (Figure 9.8). initially, the best bright colour of anthocyanin extract obtained by using methanol acidified with 0.5% trifluoroacetic acid (TFA).

The durability of coating was observed under different stability with addition of organic acid which all the absorbance values were decreased within 8 days. Tartaric acid was found to be the best stabilizer for anthocyanin to improve the colorant stability of *C. ternatea* L. The tartaric acid added colorant was mixed with PMMA and coated

onto glass slides to develop a coating system. In the liquid form, the anthocyanin-PMMA mixture added with tartaric acid showed the highest absorbance, which explained that tartaric acid can enhance the intensity of absorbance of the liquid coating mixture. On the other hand, the anthocyanin-PMMA mixture added with citric acid showed the lowest absorbance as compared to the mixture containing tartaric acid or without any organic acid (control). Thus, coating with 1% tartaric acid showed the best stability (the colour remain as same as original and without degradation).

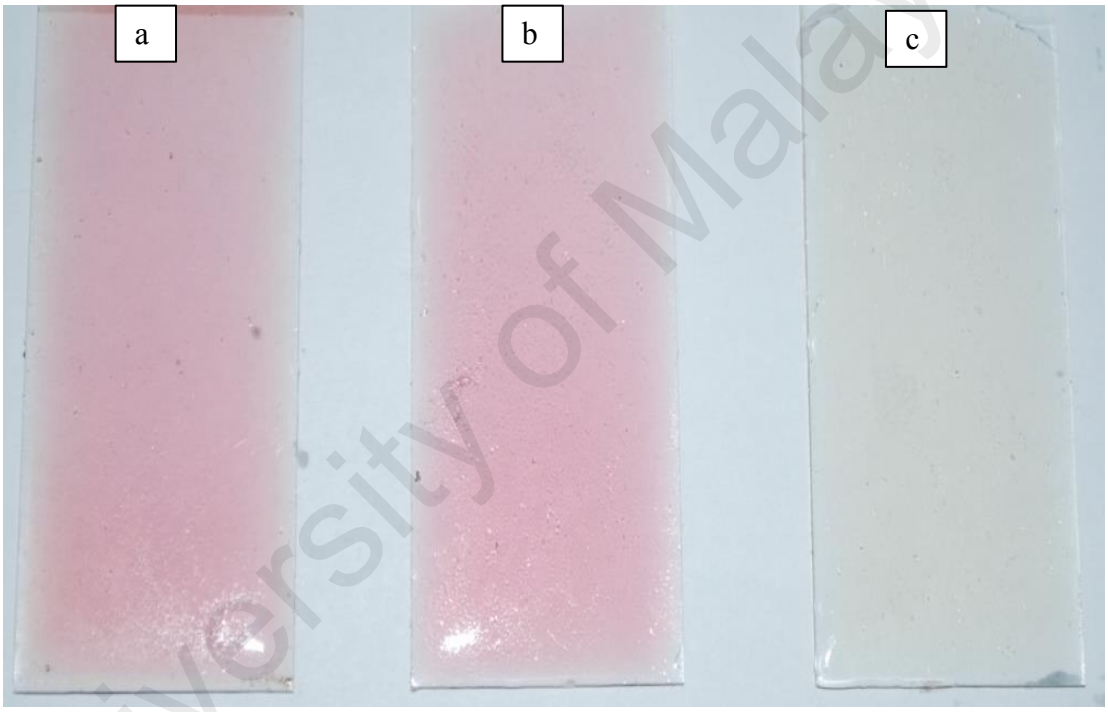


Figure 9.8 : Anthocyanin, a natural colourant from vivid blue flower petals of *Clitoria ternatea* L. onto glass slide, as coating material. (a) Extraction without organic acid. (b) Extraction with tartaric acid. (c) Extraction with citric acid.

9.4 SUMMARY OF RESULTS

1. Generally, the anthocyanin absorbance values of *C. ternatea* L. decreased when tested with higher pH, longer duration of UV radiation, higher concentrations of NaCl and longer exposure of heat (at 50±1ºC).

2. Through solvent extraction method, the best bright red colour of anthocyanin colourant was obtained by using methanol acidified with 0.5% trifluoroacetic acid (TFA).

3. The anthocyanin extraction influenced by pH, whereby extraction became red at pH 1, purple at pH 4.5, blue at pH 5.5 and cyan at pH 6.5, due to the change in the pigment's structure.

4. In the heating test, the colour of this anthocyanin extraction became darker in the oven (50±1ºC) as the duration increased.

5. Addition of 1% tartaric acid to anthocyanin-PMMA was found to be the best stabilizer to improve the colorant stability and durability of *C. ternatea* L.

6. This study showed that anthocyanins from vivid blue petals of *C. ternatea* L. can be a potential source of natural colorant for food industry and also in coating technology, especially related to green technology such as paint and varnish.

CHAPTER 10

DISCUSSION

Two important legume species from different regions were selected for this study, *Clitoria ternatea* L. (tropical perennial legume) and *Onobrychis viciifolia* Scop. (temperate perennial legume), as main sources of non-animal protein and biological nitrogen-fixation crops. Through the use of *in vitro* methods, the effect of the seasons can be eliminated and year round production can be achieved, or a new cultivar can be made commercially available more quickly and can be commercially scaled-up, and with time, production costs will reduce, therefore increasing the profit margin. Furthermore, this aids the transportation of the plantlets especially to overseas, while avoiding the risk of spread of diseases and quarantine. Therefore, the current study was focused on tissue culture for plant regeneration, callus induction, synthetic seeds production, pigment extraction, antimicrobial properties and coating technology. The successful acclimatized plants were observed under Field Emission Scanning Electron Microscope (FESEM) and histology analysis for micromorphological identification and detection of somaclonal variation, besides macromorphological study on *in vivo*, *in vitro* and *ex vitro* grown plants.

In the present study, tissue culture technique was used for both plant species for plant regeneration by manipulation and exploitation of different temperatures (18, 24 and 30±1ºC), adenine hemisulfate ($ADSO_4$, 0-100 mg/L), auxins (NAA and 2,4-D, 1.0-3.0 mg/L), cytokinins (BAP and KIN, 1.0-3.0 mg/L) and different explants (root, stem and flower bud) on solid Murashige and Skoog media (1962). The best temperature for seeds germination was at 24±1ºC, for both *C. ternatea* L. (Table 3.1) and *O. viciifolia* Scop. (Table 3.2), grown *in vivo* and *in vitro*. Generally, the results suggested that seeds germination of *C. ternatea* L. and *O. viciifolia* Scop., which originated from different climate ranges, can be achieved in the

same temperature (24+1ºC) in *in vitro* condition. Godo et al. (2011) reported that seed germination and seedling development are intrinsic characteristics for each species. According to Nikabadi et al. (2014), 98% germination rate of *Caladenia huegelii* (a temperate species) at 25ºC, whilst 63% germination rate at 20ºC on half strength MS medium. Similar with this, Rojas- Aréchiga and Vásquez-Yanes (2000) stated that favorable temperatures for germination of cacti (a desert species) are between 15 and 35 °C and the optimum temperature for germination is 25 °C, under *in vitro* condition. In the current study, the addition of 40.0 mg/L ADSO4 to MS basal media for seeds germination resulted the formation of white callus at tap roots and stems within 10-30 days, which was 5 months earlier than on MS basal alone (Table 3.3). After 20 weeks in culture condition, the root explant was the most responsive for both species. Specifically, the white callus formation from *C. ternatea* L. (Figure 3.3) was rapidly and vigorously obtained from seed germination on MS supplemented with 40.0 mg/L ADSO4 at taproot (in 10 days) and followed by stem explants (in 30 days). However, *O. viciifolia* Scop. (Figure 3.4) responded longer to MS supplemented with 40.0 mg/L ADSO4 which produced white callus only at taproot (in 20 days), while stem explants grew normally. Based on the results, the addition of ADSO4 to MS basal media gave an extraordinary respond to the roots of both species, grown at 24±1ºC. However, the growth development of *O. viciifolia* Scop. was two times faster than *C. ternatea* L. when grown at 18±1ºC. Contrary to this, Ortiz et al. (2000) stated the normal germination growth of Leguminosae *(Acacia farnesiana)* on MS supplemented with 217 µM ADSO4. Generally, MS medium supplemented with ADSO4 was to promote for shoot multiplication and *in vitro* flowering of many flowering plant species (Chang and Chang, 2003; Zhang et al., 2008;Carra et al., 2012). In addition, Vicas (2011) reported the presence of ADSO4 on MS media of *Trifolium repens* (Leguminosae) reduces the time of differentiation of the callus and of the nodules at

half (the callus formation only in 40 days) on MS supplemented with 2.0 mg/L cytokinin, 0.1 mg/L auxin and 40 mg/L $ADSO_4$, as compared to other experiments (over 70 days). In 2013, Awal et al. reported that the highest frequency of reproductive shoot regeneration (red calyx, 8.5%) was obtained within 8 weeks from explants of immature inflorescence of Begonia *x hiemalis* Fotsch cultured on MS medium supplemented with 1.0 mg/L BA and 1.0 mg/L NAA, added with 40 mg/l adenine and 3% sucrose.

In the current study of *in vitro* regeneration, the root and stem explants from 2-week-old aseptic seedlings and flower bud (intact explant) of *C. ternatea* L., were then cultured on MS media supplemented with 40 mg/L $ADSO_4$, as well as with combinations of NAA and BAP (Table 3.4) and KIN and 2,4-D (Table 3.5). The optimum regeneration medium for *C. ternatea* L.was MS media supplemented with combination of 40.0 mg/L $ADSO_4$, 2.0 mg/L NAA and 1.0 mg/L BAP with 12.03±0.12 number of shoots per explant and 8.72±1.27 number of roots per explant (Figure 3.5). Generally, *C. ternatea* L. was more responsive when cultured on $ADSO_4$-added MS media supplemented with NAA and BAP, while *O. viciifolia* Scop. Performed best on $ADSO_4$-added MS media supplemented with 2,4-D and KIN.

Other than that, *in vitro* regeneration of *O. viciifolia* Scop. was using 10-day-old root explants (with white callus). Previously, Saglam (2010) used the younger explants (7-day-old) for shoots formation from cotyledon node of *O. sativa*. On the other hand, Ozgen et al. (1998) reported shoot formation of *O. viciifolia* from stem, petiole, and leaf explants. However, to date, no report on formation of white callus on roots using MS media supplemented with combinations of $ADSO_4$ NAA, BAP, 2,4-D and KIN within 10 days. According to Sancak (1999), 26.2 shoots were obtained from embryonic axis when cultured on MS media supplemented with combination of 2.0 mg/L BAP and 0.1 mg/L IBA after 8 weeks. While Ozgen et al. (1998) obtained 12.3 shoots per explant on MS media supplemented with combination

of 20.0 µM BA and 0.5 µM NAA from stem explants. In consonance of this growth respond, *in vitro* plants need different concentration of auxin and cytokinin, since there were various types and amount of metabolites and endogenous hormones in different explants (including stem, leaf, root, flower and node) at different developmental stages (Celiktas et al., 2006).

As a comparative study, therefore all the experiments for plant regeneration of *O. viciifolia* Scop. were the same as *C. ternatea* L. using MS media supplemented with $ADSO_4$, NAA, BAP, 2,4-D and KIN (Table 3.6 and 3.7). According to Arumugam and Panneerselvam (2012), the inclusion of cytokinins and auxin caused swelling at the bases of explants over 6-10 days of culture and the addition of a cytokinins and auxin to a medium was essential to induce axillary shoot proliferation. The concentration and type of cytokinin together with auxin used significantly affected the number of shoots, number of nodes and length of shoot regeneration. Previous study on *C. ternatea* L. by Rout (2005), obtained maximum nodal cutting proliferation on MS media supplemented with NAA and BA. Based on report by Mukhtar et al. (2010), that identified that the proliferation of shoots was achieved on MS medium supplemented with various concentrations of BA, KIN and 2-iP either applied singly or in combination with NAA. The optimum medium for *O. viciifolia* Scop. was MS supplemented with combination of 40.0 mg/L $ADSO_4$, 1.0 mg/L 2,4-D and 3.0 mg/L KIN with 17.97±0.09 number of shoots per explant and 4.11±0.42 number of roots per explant (Figure 3.6). The highest shoot formation (15.33±0.51) of *C. ternatea* L. was obtained from root explants that were cultured on MS supplemented with 40.0 mg/L $ADSO_4$ and 2.0 mg/L KIN, after 20 weeks. The highest root formation (8.72±1.27) of *C. ternatea* L. was obtained from root explants that were cultured on MS supplemented with 40.0 mg/L $ADSO_4$, 2.0 mg/L NAA and 1.0 mg/L BAP, after 20 weeks, with slightly lower number of shoots (12.03±0.12) (chosen as optimum media for both shoot and root

formation). The highest shoots (17.97±0.09) and roots (4.11±0.42) formation of *O. viciifolia* Scop. were obtained from root explants cultured on the same media (MS supplemented with 40.0 mg/L ADSO4, 1.0 mg/L 2,4-D and 3.0 mg/L KIN), for 20 weeks.

Overall, the best explant for both *C. ternatea* L. and *O. viciifolia* Scop. was root explant (2-3 weeks old) for shoot and root formation, however *C. ternatea* L. was more responsive to $ADSO_4$-added MS media supplemented with NAA and BAP, while *O. viciifolia* Scop. was more suitable to be cultured on $ADSO_4$-added MS media supplemented with 2,4-D and KIN, which resulted in two times faster growth than *C.ternatea* L. Initially, after 1 week, all explants (root, stem and flower bud) of *C. ternatea* L. and *O. viciifolia* Scop. (root and stem) produced callus in all the treatments. The embryogenic and non-embryogenic callus (Figure 4.1) were determined by double staining method which influenced by age of callus, as well as type and concentrations of hormones. The embryogenic callus was distinguished by double staining method which proved that somatic embryogenesis occured in all callus cells which stained intense bright red when stained with acetocarmine (Mahmad, 2016). The highest non-embryogenic dried callus of *C. ternatea* L. (Table 4.1 and 4.2) was obtained from root explant (0.43±0.02 g, cultured on MS supplemented with 40.0 mg/L $ADSO_4$ and 2.0 mg/L KIN), followed by stem explant (0.23±0.01 g, cultured on MS supplemented with 40.0 mg/L $ADSO_4$ and 3.0 mg/L KIN) and flower bud explant (0.21±0.05 g, cultured on MS supplemented with 40.0 mg/L $ADSO_4$, 3.0 mg/L NAA and 3.0 mg/L BAP). The highest embryogenic dried callus from root explant of *C. ternatea* L. (Figure 4.2) was obtained on MS supplemented with 40.0 mg/L $ADSO_4$, 2.0 mg/L NAA and 1.0 mg/L BAP (0.31±0.02 g), which was lower compared to *O. viciifolia* Scop. (1.05±0.01 g) cultured on MS supplemented with 40.0 mg/L $ADSO_4$, 1.0 mg/L NAA and 2.0 mg/L BAP. The highest non-embryogenic dried light green callus

from flower bud explant of *C. ternatea* L. was obtained on MS supplemented with 40.0 mg/L $ADSO_4$ and 3.0 mg/L 2,4-D with 0.18±0.07g of dried callus (Figure 4.4). Generally, the responses in terms of colour and texture of callus formation based on the type of hormones, which were formed dark green and compact non-embryogenic callus on supplemented with NAA and BAP, but formed light green and friable non-embryogenic callus on MS supplemented with 40.0 mg/L $ADSO_4$ besides 2,4-D and KIN. The highest dried callus of *O. viciifolia* Scop. (Table 4.3 and 4.4) was obtained from root explant (1.44±0.02 g of non-embryogenic callus cultured on MS supplemented with 40.0 mg/L $ADSO_4$, 1.0 mg/L NAA and 3.0 mg/L BAP), followed by stem (0.73±0.02 g of embryogenic callus cultured on MS supplemented with 40.0 mg/L $ADSO_4$ and 2.0 mg/L KIN). Overall, root and stem explants of *C. ternatea* L. produced compact dark green callus, while, *O. viciifolia* Scop. formed friable light green callus. *O. viciifolia* Scop. managed to produce three times greater amount of dried callus compared to *C. ternatea* L. within 8 weeks. The embryogenic callus formed through four main different stages (globular, heart, torpedo and cotyledonary) of *C. ternatea* L. (Figure 4.5) and *O. viciifolia* Scop. (Figure 4.6). According to Kumar and Thomas (2012), optimum embryogenic callus (75 %) was induced from cotyledonary explants on MS media supplemented with 2 mg/l 2, 4-D, followed by subculturing the callus on MS medium supplemented with 2 mg/L BA and 0.5 mg/L NAA.

Through tissue culture technique, regenerated plantlets (especially for *C. ternatea* L. and *O. viciifolia* Scop.) through callus are genetically identical, disease free and mass propagation in a very short period of time without any geographical and seasonal constraints. Researchers in biotechnology and related improvements have manifested great interest over the time for the *in vitro* behavior of some species of economic value and for some species of perennial forage legumes (Varga et al., 1998, Savatti et al., 2006) such as *Onobrychis*

viciifolia, Clitoria ternatea and *Trifolium repens*. Over time, breeders have been interested in the *in vitro* behavior of theperennial forage legumes variety and in its response to certain hormonal compounds, following its capacity of regeneration, multiplication and of obtaining new *in vitro* mutations, in controlled conditions (Phillips and Collins, 1984). Theoretically, equal amount of auxin and cytokinin promotes for callus induction, but in practice it differs to a good extend may be due to the variation in endogenous level of phytohormones, as various combination and concentration of auxins and cytokinins are more effective for callus induction (Khatun et al. 2003), especially NAA, BAP, 2,4-D and KIN that have been used in the current study. For instant, combinations of BAP and NAA was proved to be the best for multiplication of shoots in *Teucrium fruticans* L. (Frabetti et al., 2009) and *Catharanthus roseus* (Swanberg et al., 2008). The effect of ADSO4 is known in the tissue cultures at many plant species and types of vegetal tissues, effect that is superior in combination with a balanced dose of cytokinin and auxin (M. Zăpârţan, 2001). The initiated callus were then allowed to grow on shoot induction medium with increasing concentration of 2, 4-D and KIN along with ADSO4 as an additives. ADSO4 was proved to be best for shoot differentiation in *Cichorium intybus* L.cv. Focus (Nandagopal et al, 2006), *Ophiorrhiza prostrate* (Beegum et al. 2007) and *Melia azedarach* L. (Hussain et al., 2009).

PGRs were commonly used in tissue culture for callus, shoots and roots induction from small piece of tissue to masspropagation, at rapid time. According to Vicas (2011), the experiment on Leguminosae (*Trifolium repens* L.) from bud explant on MS supplemented with 1.0 mg/L BA and 40 mg/L ADSO4 has stimulated a good regeneration percentage (80-90%), with a balanced number of plants (about 8 plants/explant), but also the formation of nodules (7-8/explant) along the root system, much thickened as on MS supplemented with 1.0mg/L Z, 0.1mg/L AIA and 40mg/L ADSO4 , after 40 days. Zibbu (2010) reported that high frequency

regeneration of plant via the culture of intact leaf explant of *Thevetia peruviana* (Pers.) Schum cultured on MS medium supplemented with a combination of 2.5 mg/L 2,4-D and 1.2 mg/L KIN produced dark green and friable callus after 20-28 days of inoculation. Subsequently, callus were subcultured for shoots formation for 30 days on MS medium supplemented with 2.0 mg/L 2,4-D, 1.0 mg/L KIN and 0.25 mg/L ADSO4 (3-4 shoots/explant) and 3.0 mg/L BAP (6-7shoots/explant), respectively. *In vitro* elongated shoots rooted on MS medium supplemented with 0.5 mg/L IBA. Kumar et al. (2014) reported the successfully callus induction of Leguminosae (*Solanum tuberosum* L.) from leaf explants were cultured on MS media containing 3.0 mg/L 2,4-D and 1.0 mg/L KIN, whilst the best shoot regeneration from callus (18-21 shoots/explant) was observed on MS media containing 1.5 mg/L BA and 25.0 mg/L ADSO4, after 60 days of inoculation. According to Kumar and Chandra (2014), an efficient protocol for plant regeneration through somatic embryogenesis was established from *in vivo* leaf explants of *Swertia chirayita* with the highest frequency (76%) of embryogenic callus was induced on MS medium with 0.5 mg/L 2,4-D and 0.5 mg/L KIN, after 6 weeks.

In the present study, MS medium was used as basic tissue culture medium for plant regeneration of *C. ternatea* L. and *O. viciifolia* Scop. with addition of $ADSO_4$ (play a vital role for the mass multiplication as same as other PGRs). Paques and Boxus (1987) stated that MS medium is the most suitable and most commonly for plant regeneration from tissues and callus in some plant species. It is well known that cytokinins also stimulate plant cell division and participate in the release of lateral bud dormancy, in the induction of adventitious bud formation, in the growth of lateral buds and in the cell cycle control (Gaspar et al., 1996; Gaspar et al., 2003). The benefits of ADSO4 are often only noticed when it is associated together with cytokinins such as BAP or kinetin (Van Staden et al., 2008). Arumugam et al., (2009) reported an efficient plant regeneration system was established from

immature leaflet-derived callus of *Acacia confusa* Merr, with the highest percentage of shoot regeneration response (95%) and greatest number of shoots (52.9) were obtained after the 46-day transfer of green nodular calli onto the shoot regeneration medium (McCown's Woody Plant Basic Medium, WPM) supplemented with combination of 3.0 mg/L BA , 0.05 mg/L NAA , 0.1 mg/L Zeatin and 5.0 mg/L ADSO4. Mohamed and Taha (2011) reported the micropropagation of *C. ternatea* L. on DKW medium with maximum number of shoots was achieved in DKW medium containing 1 mg/L BAP and the maximum number of root multiplication was achieved in DKW medium containing 2.0 mg/L NAA.The simulative role of ADSO4 in shoot multiplication (as resulted in Chapter 3 and 4) from different explants (such as stem, root and flower bud) as been emphasized from time to time in various plants (Dhar and Upreti, 1999; Hussain et al., 2008).

In the present study, auxins (NAA and 2,4-D), together with cytokinins (BAP and KIN), are involved in controlling morphogenesis in plant tissue culture. Different concentrations and combinations of auxins and cytokinins have different effects on the growth of explants. A balance between auxin and cytokinin growth regulators is most often required for the formation of adventitious shoot and root meristems. The concentration of each type of hormone differs greatly according to the kind of plant being cultured, the cultural conditions and the types of hormones used; interactions between the two classes of hormones are often complex, and more than one combination of substances is likely to produce optimal results. A low concentration of auxin is often beneficial in conjuction with high level of cytokinin when shoot multiplication is required. A low concentration of cytokinin (typically 0.5-2.5 μM) is often added to media containing relatively high concentration of auxin for the induction of embryogenic callus, especially in broad-leafed plants (George., 1993). Generally, when the concentration of auxin was low, root initiation was favored; whereas when the concentration is high, callus formation occurs.

The most common synthetic auxins used in tissue culture are 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (picloram, PIC). Naturally existing indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) are also frequently used. Plant tissue culture system is unique, cytokinins are commonly used in adventitious shoot bud formation, multiple shoots proliferation, somatic embryogenesis and inhibition of root formation. Cytokinins are generally considered as a critical factor for *in vitro* shoot production and there are many reports that BAP exhibits beneficial effect over other cytokinins for shoot multiplication (Dantu and Bhojwani 1987; Rao and Purohit 2006). In intact plants, they particularly stimulate protein synthesis and participate in cell cycle control. Added to shoot culture media, these compounds overcome apical dominance and release lateral buds from dormancy.

In the current study, efficient plant regeneration system via somatic embryogenesis of *C. ternatea* L. and *O. viciifolia* Scop. were successfully developed using $ADSO_4$, NAA, BAP, 2,4-D and KIN from root and stem explants. For successful callus induction, factors such as type of explants, PGRs, culture media and culture conditions are very important (Yeoman and Yeoman, 1996; Ali et al., 2009). According to Arumugam and Panneerselvam (2012), embryogenic system offers an ideal tool for *in vitro* production and selection of transgenic plants due to non-availability of efficient regeneration system in many grain legumes. Initially, somatic embryos will develop on one part of the explant as globular stage (protuberances). This continued with individual embryos enlarged into distinct bipolar structures and the somatic embryos matured through globular, heart, torpedo and cotyledonary shaped stages. The percentage of embryogenesis was significantly influenced by the concentration of the plant growth hormones used in the media (Pathi et al., 2013). According to Amirato (1983) and Sato et al. (1993), the regenerations of *in vitro* plants via somatic embryogenesis will produce a

high number of regenerants. Karamian and Ranjbar (2008) reported the maximum frequency of embryogenic callus induced from hypocotyl explants of *Onobrychis subnitens* with addition of 1.0 mg/L 2,4-D. According to Kumar and Thomas (2012), optimum embryogenic callus (75%) was induced from cotyledonary explants on MS medium supplemented with 2.0 mg/L 2,4-D, followed by subculturing the callus on MS medium supplemented with 2.0 mg/L BA and 0.5 mg/L NAA, after 45 days. Vidal et al. (2014) reported that there were no significant differences relative to the type of explants and auxin concentrations in the frequency of callus with embryos at 55-75 days of evaluation, as well as the number of embryos per callus was not influenced by the type of explants, but the types and concentrations of the hormones. The cotyledonary stage was characterized by the formation of multi-cotyledonary embryos containing two cotyledons. According to Dornelas et al. (1992), the base of the embryonary axis of somatic embryos of multicellular origin tends to be widely linked to the explant, without the formation of the suspensor or an equivalent structure. Hill et al. (1989) stated that the highest frequency of callus of *Hosta sieboldiana* from immature florets explant on MS supplemented with 5.4 µM NAA and 4.4 µM BA, whilst the highest shoot initiation at 0.4 µM BA.

By using similar hormones (BAP and 2,4-D), Garshasbi *et al*. (2012) reported that the callus induction from leaf cotyledon and apical meristem explants produced the greatest callus in existence of two hormones with high ratio 2,4-D to BAP. In a medium which lacks 2,4-D, the smallest callus produced from leaf cotyledon explant and no callus was produced from apical meristem. In his conclusion, the increase of 2,4-D ratio to BAP in each explant raised callus production of *Onobrychis* sp. Therefore, differences in explant used could affect the optimum medium to induce callus. Based on Karamian and Ranjbar (2008) studies, somatic embryos and adventitious buds were induced simultaneously when

embryogenic callus from MS medium supplemented with 2,4-D and BAP were transferred to MS medium with NAA and BAP . The highest frequency of embryoids and maximum adventitious bud frequency was obtained. The studies also conducted on the effect of NAA hormone on immature inflorescences, apical-axillary meristems, internode, leaf and petiole of *Onobrychis* sp. by Celiktaz *et al*. (2006).

Based on Arumugam & Panneerselvam (2012) study, the maximum callus initiation was observed by adding 2,4-D (1.0 mg/L) and KIN (0.5 mg/L) using leaf explants of *Clitoria ternatea*. The combination of plant growth regulators such as 2,4-D and KIN induced best callus in all the explants of *C. ternatea* L. compared to other hormonal combination (Arumugam and Panneerselvam, 2012). However, according to Shahzad et al. (2007), various growth regulators gave different effects on *C. ternatea* L. but there were no effects showed on the explants when cultured on MS basal media. According to Arumugam & Panneerselvam (2012) study, the inclusion of cytokinins and auxin caused a swelling at the base of explants over 6-10 day of culture and the addition of cytokinins and auxin to a medium was essential to induce axillary shoot proliferation. Mukhtar, (2010) identified that the proliferation of shoots of *C. ternatea* was achieved on MS medium supplemented with various concentrations of BA, KIN and 2-Ip, either applied singly or in combination with NAA. Of all the cytokinins, BA, KIN and 2-Ip tested on the MS medium, BA (2.5 mM) was optimum for inducing the maximum number of shoots (9.8 ± 0.86). The highest efficacy for shoot proliferation (13.2 ± 0.31) was achieved with a combination of 2.5 mM BA and 1.0 mM NAA. The best condition for rooting was observed on half strength MS medium augmented with 1.5 mM indole-3-butyric acid. In previous study on tissue culture of Leguminosae (Fabaceae), a few species were exposed to nutrient media supplemented with combinations of auxins, cytokinins and $ADSO_4$ such as *Acacia confuse Merr*. (Arumugam et al.,

2009) and *Trifolium repens* (Vicas, 2011), To date, there is no report on *in vitro* regeneration from root explant of *C. ternatea* L. and *O. viciifolia* Scop. cultured on MS supplemented with combinations of NAA, BAP and $ADSO_4$, as well as 2,4-D, KIN and $ADSO_4$. Due to limitation of seeds, especially for imported species (*O. viciifolia* Scop.), synthetic seeds production was another alternative method for mass propagation (CHAPTER 5). Therefore, previous treatments were important for callus formation, furthermore for the synthetic seeds production and antimicrobial activities. The optimum concentration for the formation of encapsulation matrix for callus was 3.0% (w/v) sodium alginate ($NaC_6H_7O_6$) and soaked in calcium 100 mM (w/v) chloride dehydrate ($CaCl_2.2H_2O$) solution for 30 minutes (Table 5.2). Synthetic seeds from embryogenic callus (6-week-old) of *C. ternatea* L. (Figure 5.2) was germinated better (24.9±0.8 to 30.0±0.0 number of seeds) as compared to *O. viciifolia* Scop. (Figure 5.3) with 19.7±2.3 to 30.0±0.0 number of seeds, in 90 days. The viability of synthetic seeds of *C. ternatea* L. was reduced after 45 days of low temperature storage (4±1ºC) with 28.2±0.6 number of seeds, but still with 100% survival rate, while, the germination of *O. viciifolia* Scop. was reduced for every 15 days interval.

Previously, Kumar & Chandra (2014) reported on synthetic seeds produced by encapsulating of torpedo stage embryos in sodium alginate (4% w/v) gel, dropped into 100 mM calcium chloride solution and germinated (84%) on MS medium supplemented with 1.0 mg/L BA and 0.5 mg/L NAA. Synthetic seeds can also be used as artificial propagules for plants which reproduce asexually. Synthetic seeds consist of viable plant parts and artificial endosperms which can be germinated when necessary. The synthetic seeds can be stored under low temperature (4°C) and germinated throughout the year and independent of seasons. Synthetic seeds can be produced for elite genotypes of this species to ensure the uniformity and clone nature of the offsprings cultivated in the field. The most outstanding advantage is the capability of

small pieces of vegetative tissues to produce hundreds or more artificial propagules through plant tissue culture technology. According to Kumar and Thomas (2012), the highest seeds germination (92%) from synthetic seeds of *C. ternatea* L. from leaves explant was observed on MS medium supplemented with 2.0 mg/L BA and 0.5 mg/L NAA. Rao and Purohit (2006) reported that the encapsulated shoot tips can be handled like a seed and could be useful in minimizing the cost of production as 1 ml of medium is sufficient for encapsulation of a single shoot tip compared to 15-20 ml for conversion of shoot tips into plantlets. As compared to suckers, encapsulated shoot tips present as inexpensive, easier and safer material for germplasm exchange, maintenance and transportation. The development of artificial seed production is effective and acts as an important alternative method of propagation in several commercially important plant species with high commercial values. Synthetic seed production has many advantages in storage over conventional propagation. Consequently, genetic uniformity and stability of the plant could be maintained due to the sterility. As the result, plants could be produced in large scale with high volumes. At the same time, cost would be cheaper.

In order to adapt and grow well under natural environment with normal growth performances, the regenerated plants (plantlets) were transferred to selected soils for acclimatization. The highest survival rate (91.07±0.96%) obtained from plantlets of *C. ternatea* L. (Table 6.1), on mud soil (pH 4.26±0.12), while, *O. viciifolia* Scop., (71.58±1.27%) performed best on top soil (pH 5.14±0.12). After 7 months being acclimatized, the *ex vitro* plants of *C. ternatea* L. was shorter (39.16±0.28 cm), which was about half as compared to the *in vivo* plant height (95.03±0.83), but the quality of leaves (2.11±0.11 cm) and flowers (9-16 flowers per plant) were better, besides nodules formation were observed with 25-40 nodules per plant (Table 6.2). After 7 months of acclimatization in the green house, the regenerants of *C. ternatea* L. (Figure 6.1) showed similar growth

characteristics as mother plants, whilst, *Onobrychis viciifolia* Scop. (Figure 6.2) died after 2 months being exposed to Malaysian temperature (30±1ºC) with shorter stems (6.98-8.00 cm) and smaller leaves (0.20-0.40 cm), which was about 10 times stunted than *C. ternatea* L. (Table 6.3 and Figure 6.4). In acclimatization process, the regenerated plants usually showed low survival rates when acclimatized due to the loss of water, inefficient stomata functions, poorly developed cuticle wax on leaves produced and many others inefficiency. Medium or substrates used for transplanting purposes must be sterilized. A chilling treatment before transplanting may improve survival rate and growth of plants. Plantlets transferred directly to greenhouse had 100% mortality, primarily due to poor growth performance (Habib et al., 2013).

A gradual acclimatization is a critical step of transplanting and it significantly affects performance of transplanted plants. After being transplanted, *in vitro* plants were very sensitive to fungi and bacteria, which spread quickly among the plantlets if a nonsterile environment exists. Therefore, individual acclimatization in separate pots or multi-plots was suggested to reduce disease development and spread (Albers and Kunneman, 1992). Low temperature with high humidity also may benefit transplanting procedure. Plantlets were transferred to sterile clay soil and uncovered in culture rooms for 21 days. Micropropagated plants need sometime for adaptatation to the environment, so the culture vessels need to be opened a few days before *in vitro* plantlets are transferred to the natural environment. It is necessary to treat plantlets 2–3 months before transplanting in order to improve survival rate. The performance of transplanted plants largely depends on acclimatization procedures involving adaptation of plantlets to *ex vitro* conditions of significantly lower relative humidity and higher light intensity. During *in vitro* culture, plantlets grow in high humidity and low irradiance and plants that developed under lower relative humidity have fewer transpiration and translocation problems *ex vitro* and

persistent leaves that look like normal ones. After transplanting (acclimatization), about 75% of plantlets survived (Liu et al., 2002; Luo et al., 2004b).

According to Sorensen and Sessitsch (2007), in Leguminosae, the symbiotic association with N-fixing rhizobia bacteria which live in small growths attached to the plant roots called nodules. Most perennial legumes showed finger-like shape of nodules, including *C. ternatea* L. and *O. viciifolia* Scop., which contribute to biological nitrogen fixation (change N_2 to NH_3) and in return, the plant contributes the nutritional and energy (photosynthate or photosynthesis-derived sugar) for the bacteria. Nodules on perennial legumes are long-lived compared to annual legumes, which do not depend on growing season. Nitrogen fixation by legumes ranged from 25-75 lb of nitrogen per acre per year in a natural ecosystem compared to several hundred pounds in a cropping system (Frankow-Lindberg and Dahlin, 2013; Guldan et al., 1996). Perennial forage legumes such as alfafa, sweet clover, true clover and vetches, may fix 250-500 lb of nitrogen per acre, which occasionally respond to nitrogen fertilizer at planting or immediately after a cutting when the photosynthate supply is too low for adequate nitrogen fixation (Aranjuelo et al., 2009). However, results from Chapter 6, revealed only *C. ternatea* L. was found to form nodules after 2 months being acclimatized on black garden soil. No nodule was observed from *O. viciifolia* Scop. The nodules of *C. ternatea* L. showed the colour changed from white to orange within 2 weeks. Basically, white or grey nodules indicating that the nodules are at the young stage and not yet function in fixing nitrogen. Gradually, the colour change to pink or reddish (matured), indicating fixation has started with the presence of leghemoglobin that controls oxygen flow to the bacteria. Unfortunately, the plant will discard the pink or reddish nodules that changed to green (no more N-fixing activity). This probably because of the inefficient bacteria, poor plant nutrition (phosphorus, potassium, zinc, iron, molybdenum and cobalt) or pod filling. Moreover, environmental stresses

including temperatures and water availability (irrigation) will also reduce N-fixing activity. This bacteria activity and soil can be improved by bacteria inoculation, fertilization and irrigation. However, most of the legumes preferred to fix nitrogen on their own rather than getting it from nitrogen fertilizer (Walley et al., 1996), subsequently return the N to soil through entire biomass as green manure. Thus, nodules from legumes contributed to the soil improvement with low and easy maintainance, naturally.

Macromorphologically, *C. ternatea* L. and *O. viciifolia* Scop. grew healthier in *in vitro* condition (at low temperature and high humidity) with smaller in size (miniature) and without any infection by bacteria or fungi (as resulted in Chapter 3 and 4). In Chapter 6 and 7, the macromorphology and micromorphology of *in vivo* and *in vitro* grown *C. ternatea* L. and *O. viciifolia* Scop. were compared. For *C. ternatea* L. (Table 6.4), initially, under *in vivo* condition, black matured seeds (0.4-0.6 cm) were germinated faster (3-5 days) under full sun than shade area (7-14 days). The plants were grown vigorously with oval in shape and light green of twiners, dark green of leaves (0.9-1.3 cm) and woody climber stems (0.4-0.7 cm). However, the macromorphology of regenerated *C. ternatea* L. were performed differently (miniature in shape and size). In germination stage on MS basal medium, the seeds were germinated as fast as (3-6 days*), in vivo* grown seeds. In the optimum medium (MS supplemented with PGR), the leaves were a bit round in shape and smaller (0.3-0.9 cm). The stem (0.1-0.2 cm) remain as green and there was no woody climbers observed. After 7 months under *in vitro* conditions, the plants were transferred to the green house.

In *in vitro* cultures, auxins are usually used to stimulate callus production and cell growth, to initiate shoots and rooting, to induce somatic embryogenesis, to stimulate growth from shoot apices and shoot stem culture. The auxin NAA and 2,4-D are considered to be stable and can be stored at 4^{o}C for several

months (Gamborg *et al.*, 1976). They affect cell elongation by altering cell wall plasticity. Other than that, also stimulate cambium, a subtype of meristem cells, to divide and in stems cause secondary xylem to differentiate. In in vitro condition, auxins act to inhibit the growth of buds lower down the stem (apical dominance) and also to promote lateral and adventitious root development, whilst, cytokinins including BAP and KIN, also help delay senescence or the aging of tissues, are responsible for mediating auxin transport throughout the plant, and affect internodal length and leaf growth (Sipes and Einset, 1983). Since, the *in vitro* grown plant were transferred to natural environment after 7 months, therefore, the *ex vitro* grown plants still influenced by the PGRs (at the beginning of the acclimatization stage with small leaves and stunted).

The regenerated plantlets were removed from the culture containers carefully. The roots were washed to get rid of the gelrite. For the first 3 weeks, plantlets of *C. ternatea* L. were acclimatized on mud soil in the culture room at 24±1ºC for adaptation or acclimatization process. Then, the plantlets were transferred to the green house at 30±1ºC. The macmorphological characteristics in Table 6.2 and Figure 6.1 showed the differences in size of stem height and leaf width, number of flowers and number of nodules between *in vivo*, *in vitro* and *ex vitro* grown plants of *C. ternatea* L. At the same age of 7-month-old, *in vivo* plants showed the highest stem (95.03±0.83 cm**)**. Even though, the *ex vitro* plants was shorter (39.16±0.28 cm), which was about half as compared to the *in vivo* plant height , however, the quality of leaves (2.11±0.11 cm) and flowers (9-16 flowers per plant) were the best among the three growth conditions. Moreover, in this study, only *ex vitro* plants managed to form nodules (25-40 nodules per plant) which started after 6 weeks being transferred to soil consist nodules of white and orange nodules attached to the secondary roots. A week later, the first twiner was formed. After 12 weeks, the plantlets started to flower and fruiting (after 3 months) which were in shorter time as compared to the *in*

vivo plants (5-6 months). The *in vivo* primary roots were long and large (8.7-15.2 cm length and 0.2-0.3 cm width) and the young or white nodules (10-35) were observed at the secondary roots. The white nodules change to orange nodules within 6-7 days. The *ex vitro* primary roots were shorter (7.2-12.5 cm length), but as width as *in vivo* grown plant (0.2-0.3 cm width). Nodules only observed after 2 months being acclimatized, but with small quantity (8-13 nodules). Finally, the plants growth and structure were the same as the mother plant (*in vivo*). For *O. viciifolia* Scop. (Table 6.3 and Figure 6.4), the *in vivo* and *ex vitro* plants only survived for 3-4 weeks, due to the poor adaptations to the high temperature (30±1ºC). The *in vivo* leaves showed darker green and larger (0.45±0.31) than *in vitro* (0.21±0.09) and *ex vitro* (0.27±0.07) grown plant, however with minimum number of leaves (3-6). Although, the survival rate was higher (71.58±1.27) on topsoil at the beginning of the experiment, however, after 4 weeks the plants failed to adapt and became weak and died. The *in vitro* regenerated plants survived up to 8-10 months, whilst *ex vitro* only for one month.

Observations of micromorphological through FESEM and histology analysis arerequired to compare the characteristics of leaves of *in vivo* and *in vitro* grown plants of *C. ternatea* L. and *O. viciifolia* Scop. (CHAPTER 7). Hence, the *ex vitro* plants were compared to *in vivo* plant on micromorphological distribution of stomata and trichomes of the leaves of *C. ternatea* L. (Figure 7.1 and 7.2) and *O. viciifolia* Scop. (Figure 7.3 and 7.4) using Field Emission Scanning Electron Microscope (FESEM), grown *in vivo* and *in vitro*. The FESEM micrographs on 4-week-old leaf of *C. ternatea* L. showed abaxial surface of *in vivo* grown leaf with stomata in a shallow pit (to reduce wind speed and to slow transpiration water loss), while, the abaxial surface of *in vitro* grown leaf contains rough texture that caused by the wax fibers. The FESEM analysis of *O. viciifolia* Scop. showed the presence of non-glandular trichomes with sharp-point end. Werker (2000) reported that the

pointed, hairy structure of non-glandular trichomes located on the leaf veins on the abaxial surface of leaves, at the tips of the abaxial surface of corollas, and at the bases of the adaxial surface of corolla tubes may serve as a mechanical barrier against various external factors such as herbivors and pathogens, UV-B radiation, extreme temperatures, and excessive water loss by transpiration. Generally, the micromorphology and distribution of non-glandular trichomes on leaf surface have brought to knowledge about the function as protection against extreme environment.

In the present study, the stomatal variation is analyzed using FESEM and light microscopy (histology). This study also indicates that stomatal and trichomes characteristics are valuable taxonomic traits, which can be utilized to address the taxonomic issues within the genus or family. The importance of micromorphological features for the taxonomic consideration of angiosperms is recently mounting up (Parveen et. al., 2000). Micromorphological parameters of different plant parts have been used as aids in the taxonomical recognition of species (Kathiresan et. al., 2011). Studies are conducted in many families on the basis of the leaf epidermis to delineate taxa (Albert and Sharma, 2013; Aworinde et. al., 2014). Size, distribution, and frequency of stomata have been found to be specific to taxa and are used as significant parameters in taxonomy as well as in elucidating phylogeny (Ahmed, 1979; Rajagopal, 1979; Idu et. al., 2000; Barkatullah et. al., 2014). Stoma, the turgor operated valve is significant in discriminating the taxa at any taxonomic levels. Hayat et. al., (2010) constantly reaffirmed that micromorphological features of plants could be exploited in the biosystematics in the scenario of modern technological revolution. Further, diversity in terms of shapes of epidermal cells, stomatal size, its orientation and trichome nature and vascular bundles distribution are all pivotal in systematics. These characteristics have been employed in many genera to solve some intrinsic taxonomic issues or to

contribute to increasing taxonomic database at species and even at family levels. Stomata were initially evaluated by Stresburger (1866) followed by Vesque (1889) to categorize them based on subidiary cells as well as their ontogeny in to four classes. Twenty five stomatal types are recognized based on leaf epidermal arrangement near the guard cells in dicots (Vishal et. al., 2012). Meanwhile, Stace (1984) recognized thirty one diverse types of stomata among seed plants. Stomatal index on leaf surfaces varies greatly among various species of plants. Usually, the lower epidermis of the leaf show increased number of stomata than the upper side. Reports suggests that the stomatal number may vary from zero on the apple leaf upper epidermis to 58,140 / square cm of black oak leaf lower epidermis. According to Patel and Inamdar (1971), stomatal development varies during the life history (stage and age of the leaf) of a species. Stomata are mostly anomocytic type, usually present on both surface of the leaves. Differences in shape, size, distribution and the orientation of stomata have been observed.

The present study also compared the micromorphological characteristics of *in vivo* and *in vitro* grown plants of *C. ternatea* L. and *O. viciifolia* Scop.. There were little differences in histological, anatomical and ultrastructural characteristics between *in vivo* and *in vitro* grown plants of *C. ternatea* L., especially for the textures and sizes of the leaves. However, *in vitro* leaves were found to be smaller compared to *in vivo* leaves, based on FESEM and light microscope examinations. This phenomenon could be due to the differences in the conditions and growth environments of the plants especially in intact plants during sunlight exposure. Hence, after 6 months of acclimatization in the green house, the regenerants showed almost similar growth characteristics as the mother plants (with same morphology, histology, anatomy and ultrastructural features), implying that no somaclonal variation occurred during *in vitro* process and growth of this species. According to Pathi et al. (2013), regenerants derived from somatic embryos,

showed morphological and growth characteristics similar to those of seed-derived plants. Taha et al. (2011) reported that SEM data of *in vivo* and *in vitro* grown *Platycerium coronarium* revealed similar ultrastructures of both types of leaves, whereby the presence of multicellular trichomes on both the abaxial and adaxial surfaces, as sunken stomata also detected on the abaxial surface of the leaves. Environmental factor especially in the degree of sunlight exposure caused the differentiation of structure of palisade and cuticle cells between *in vivo* and *in vitro.* According to Taha and Haron (2008), differences were found in the number of layers of palisade cells and the presence or absence of epicuticle layer in *Murraya paniculata* (Jack) Linn, grown *in vivo* and *in vitro*.

The comparison of structural features between *in vitro* cultured plants and *in vivo* grown plants under FESEM and histology analysis was also done. *In vitro* cultured plants have bigger stomata and less wax compare to the *in vivo* cultured plants. *C. ternatea* L. has anomocytic stomata and convex cell shape of the epidermal cells with epicuticular wax crystals (Figure 7.5). The *in vitro* cultured plant stomata were slightly open while the *in vivo* cultured plant stomata were closed. Stomata present on both surfaces of the leaf. After undergoing powder microscopy test, it showed that *C. ternatea* L. contains epidermal cells with paracytic stomata, fragments of trichomes with warty cuticle and wavy thin walled. It also showed groups of spongy parenchyma and palisade cells, fibers, veins and epidermal cells. The closed stomata might be due to the FESEM method itself in which the sample was dehydrated in order to be seen. The sample might be under conditions of acute water deficit, hence, the stomata closed in order to conserve the water so that the dehydration did not occur. The stomata pores closed also might be because of excessive water loss occured during the process. *In vitro* culture plant has bigger stomata compared to *in vivo* stomata. This may be because *in vitro* culture plant gets sufficient nutrients and does not face any stress condition. While *in vivo* culture

plant get nutrients solely from garden soil and need to compete with other organisms in order to live. The *in vitro* culture plant also has slightly thick leaf as compared to *in vivo* culture plant. Furthermore, *in vitro* culture plant has less wax compared to intact plants. This might be because of *in vitro* culture plant was cultured under sterile conditions and the wax layers may not developed well as in *in vivo* plants. The study on leaf gross morphology and structural features under FESEM showed that *C. ternatea* L. has anomocytic stomata and convex cell shape of the epidermal cells with epicuticular wax crystals. Generally, during acclimatization, transpiration rate gradually decrease because stomata regulation of water loss becomes more effective, therefore, cuticle and epicuticular waxes will develop (Baroja et al., 1995).

Trichomes (hairs) are unicellular or multicellular outgrowths that originate from the aerial epidermis and vary in morphological features, location and mode of secretion. Glandular trichomes are associated with the production of chemicals that provide defense against herbivores and pathogens. It has been suggested that non-glandular trichomes serve various functions in plants, including reducing the heat load, reflectance of UV light, provide protection from insects and herbivores, increase tolerance to freezing and maintain water balance in leaves (Werker, 2000; Mauricio and Rausher, 1997). The non-glandular trichome is supported by a basal cellular pedestal. It has been reported that the basal cellular pedestal provides mechanical support and serves as a point for the attachment of trichomes to the epidermis (Ascensao et al., 1999). The stalk of the non-glandular trichome is densely covered with cuticular warts, which could be indicative of leaf maturity and which may be involved in helping the hairs stay free of dust by promoting cleaning during rainfall; the so called ‘Lotus effect’ (Nosonovsky and Bushan, 2007). The density of non-glandular trichomes is an adaptation which used to limit incoming UV light and thus protect vascular tissues in leaf. The rough texture of leaf is caused by the presence of wax fibres (waxy cuticle). Stomata are small openings found in

epidermis of green aerial parts, especially leaves of the plants. Usually, stomata are anomocytic type, present on the upper (adaxial) and lower (abaxial) surfaces of the leaves. According to Wafa et al. (2016), the *in vivo* grown leaves of *Canna indica* L. had more stomata compared to *in vitro* grown leaves. Rasmussen (1981) classified the stomata into different types on the basis of structure and shape of neighbouring epidermal or subsidiary cells, which helped in performing physiological functions like photosynthesis, respiration and transpiration usually present on both surfaces of the leaves.

Besides FESEM, histology analysis based on the ultrastructural features from leaves of *C. ternatea* L. (Figure 7.9) and *O. viciifolia* Scop. (Figure 7.10), grown *in vivo* and *in vitro* were examined after 5 months. The adaxial (upper epidermis) from *in vivo* grown leaf of both species showed the presence of cuticle which affected by the sun exposure, besides providing protection against desiccation and pathogens. The clearer open and closed stomata were observed at abaxial (lower epidermis) which control the exchange of gases and water vapor between the leaf cells and the atmosphere. The stomatal organization consists of two subsidiary cells, one on each side of the guard cell pair. Beneath the adaxial is a layer of vertically elongated palisade mesophyll cells (PM), which packed with chloroplasts (small circular bulges within many cells) and large air space. The lower half of the leaf contains the spongy mesophyll which loosely arranged network of cells of irregular shape and large air space. Overall, the ultrastructural features of *in vivo* leaf showed more air space in palisade and spongy mesophyll layer, while the *in vitro* leaf with compact cells.

Due to the confirmation true-to-type characteristics of *C. ternatea* L. and *O. viciifolia* Scop by FESEM and histology analysis, therefore, the sterility conditions were potentially to be exploited for the pure extraction of pharmaceutical products, such as for antibacterial and antifungal properties (as resulted in CHAPTER

8). *C. ternatea* L. is also known as medicinal plant and widely used for eye, throat, skin and ulcer treatments, as well as for antimicrobial activity. In the present study (Table 8.1 and Figure 8.2) results showed that the highest inhibition zone (16.00 mm) was obtained from *in vivo* leaves extract (mother plant) of *C. ternatea* L. and had activity against both (double function) *Bacillus subtilis* and *Escherichia coli*, comparable to *in vitro* leaves extraction as anti-*bacillus subtilis* (15.00±0.56 mm), while callus extraction as anti-*Escherichia coli* (15.00±0.37 mm).

On the other hand, the highest inhibition zone (15.00±0.55 mm) for antifungal activity of *C. ternatea* L. (Table 8.2 and Figure 8.3) obtained from *in vivo* leaves extraction against *Candida albicans*, as well as by *in vitro* leaves extraction (13.00±0.57 mm). Comparing between *in vivo* and *in vitro* leaves extracts, callus performed best in inhibiting *Fusarium* sp. (14.00±0.46 mm). Petals extraction were also subjected for antimicrobial activity and compared with non-embryogenic callus that derived from flower bud cultured on MS supplemented with 40.0 mg/L $ADSO_4$ and 3.0 mg/L 2,4-D. Overall, both *in vivo* and *in vitro* ethanolic extracts from vivid blue flowers of *C. ternatea* L. showed the best antibacterial activity against *Bacillus subtilis*, 11 mm and 10 mm inhibition zones, respectively (Table 8.2 and Figure 8.3). However, different antifungal activity was detected from *in vitro* ethanolic callus extract (12 mm) against *Trichoderma* sp., contrary to *in vivo* ethanolic extract (10 mm) against *Fusarium* sp. (Table 8.4 and Figure 8.5). However, aqueous extract of petals showed no inhibition zone to tested bacteria and fungi. Mohd-Joffry et al. (2012) reported that flowers contained anthocyanin pigments especially malvidin-3,5-diglucoside in the aqueous extract. For *O. viciifolia* Scop., the highest inhibition zone (17.00±0.65 mm) was found in *in vivo* leaves extracts (Table 8.3 and Figure 8.4) against *Escherichia coli*, while for callus extract, the inhibition zone was 15.00±0.47 mm. All extractions of *O. viciifolia* Scop. gave positive response as antifungal activity to both

Fusarium sp. and *Candida albicans* including *in vivo* leaves (18 mm), *in vitro* leaves (11 mm) and callus (15-16 mm).

Due to the increasing demand for medicinal and nutritive forage purposes of *C. ternatea*, the United State Development Agency (USDA, 2014) intends to conserve *C. ternatea* L. along with 16 other leguminous species with potentially useful phytochemical compounds (Morris, 1997). *C. ternatea* L. is widely used for eye, throat, skin and ulcer (Malabadi and Nataraja, 2001), as well as for phytochemical substances. The phytochemical investigations revealed the presence of saponins, carbohydrates, alkaloids, proteins, anthroquinones and phytosterols, which are used as diuretics, antihelmintic, antidiabetic, antipyretic and brain tonic (Kirtikar and Basu, 1981). All parts of *C. ternatea* contain peptides called cliotides that have potent anti-microbial properties against *Eschericia coli* (Nguyen et al., 2011). Antimicrobial activity of the callus extract can vary between differentiated and undifferentiated cells depending on the biocompounds production (Ahmed et al., 2010). 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 the top of the soil (up to 15 cm depth) and play an important role in gas cycles (such as nitrogen fixation), while fungi (*Fusarium* sp., *Candida albicans* and *Trichoderma* sp) decaying organic substances that add cellulose and inorganic substances into the soils. Pietikäinen et al. (2000) reported that the optimum growth for bacteria and fungi are at 25-30ºC and responsible for infection in plants (losses in agricultural industry), animals and human (can caused diarrhea and skin infection). Plants can produce antifungal compounds to protect themselves from biotic attack that could be essential for fungi infection resistance (Wotjaszek, 1997). Carbendazim (as positive control) was used as antifungal. According to Grzegorczyk et al. (2007), the compounds in *in vitro*

plants had higher bioactivity than in *in vivo* plants. Antimicrobial activity differs in *in vivo* and *in vitro*, probably due to the inherent characteristics of the fully grown plants and the maturity of its chemically active constituents.

In the present study, differences of antibacterial and antifungal activities were observed from the ethanolic extracts of *C. ternatea* L. and *O. viciifolia* Scop. These could be due to the differences in the chemical composition as well as in the mechanism of action of the bioactive constituents (Cowan, 1999). Generally, phytochemical screening revealed the presence of several classes of secondary metabolites such as alkaloids, polyphenols, flavonoids, anthraquinones, coumarins, saponins, tannins, triterpenes and steroids. Several molecules belonging to these classes were found to be active on pathogenic microorganisms (Tsopmo, 2013). Medicinal and herbal plants which contain components of therapeutic have been used as remedies for human diseases for centuries. Plants can produce antifungal compounds to protect themselves from biotic attack that could be essential for fungi infection resistance (Wotjaszek, 1997). Plants also are rich in a wide variety of secondary metabolites polyphenols, such as tannins, terpenoids, alkaloids and flavonoids, which have been demonstrated to have *in vitro* antimicrobial properties (Gonzalez-Lamothe et al., 2009). Thus, based on the results in Chapter 8, *C. ternatea* L. and *O. viciifolia* Scop. may used in the treatment of infections including multi-resistant bacteria and fungi. The highest inhibition zone (16.00 mm) was obtained from *in vivo* leaves extraction (mother plant) of *C. ternatea* L. against both *Bacillus subtilis* and *Escherichia coli*. In single function, *in vitro* leaves extraction can be proposed as anti-*bacillus subtilis* (15.00±0.56 mm), while callus extraction as anti-*Escherichia coli* (15.00±0.37 mm). The highest inhibition zone (17.00±0.65 mm) was from *in vivo* leaves extraction of *O. viciifolia* Scop. against *Escherichia coli*. While, the ability of callus extraction (15.00±0.47 mm) was comparable to mother plants (16.00±0.61 mm) against *Staphylococcua aureus*.

The highest inhibition zone (15.00±0.55 mm) of antifungal activity of *C. ternatea* L. obtained from *in vivo* leaves extraction against *Candida albicans*, as well as by *in vitro* leaves extraction (13.00±0.57 mm). Furthermore, comparing among *in vivo* and *in vitro* leaves extraction, callus performed the best in inhibition of *Fusarium* sp. (14.00±0.46 mm). All extractions of *O. viciifolia* Scop. (*in vivo* leaves, *in vitro* leaves and callus), gave positive response as antifungal activity to both *Fusarium* sp. and *Candida albicans*. However, the highest inhibition zone (18 mm) by *in vivo* leaves extraction, followed by callus extraction (15-16 mm) and *in vitro* leaves extraction (11.00 mm). Both species (*C. ternatea* L. and *O. viciifolia* Scop.), responded negatively to antifungal activity against *Trichoderma* sp. However, *O. viciifolia* Scop. Was detected as anti-*Fusarium* sp. and anti-*Candida albicans*. Generally, the present results verified that the presence of antimicrobial activity against bacteria (*Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*) and fungi (*Fusarium* sp., *Candida albicans* and *Trichoderma* sp.) varied with different explants source (callus and leaves) of both species, grown *in vivo* and *in vitro*.

All plants produce peptides for antimicrobial control (Kokoska et al., 2002). These antimicrobials represent a fertile untapped source of natural compounds that can be used as therapeutic agents (Zasloff, 2002). The growing problem of resistance to conventional antibiotics and the necessity for new antibiotics has stimulated an interest in the development of antimicrobial peptides (AMPs) as human therapeutics (Maróti, 2011). AMPs are peptides that can kill microorganisms, and they often exhibit a broad spectrum of activity against gram- positive and gram-negative bacteria (Kamatou et al., 2005). To date, this is the first report on antimicrobial activity from vivid blue flower explant of *Clitoria ternatea* L., which successfully observed the antibacterial activity (both *in vivo* and *in vitro* extracts against *Bacillus subtilis)*, however different antifungal activity was detected,

whereby the *in vitro* extract was against *Trichoderma* sp., contrary to *in vivo* extract that was against *Fusarium* sp. Effective, safe and cheap medicinal agents from plants may appear as potential alternatives for controlling microbial infections particularly the resistant cases. An 80% methanol solution is the most commonly used reagent for isoflavone extraction in the literature, as reported by Kulling, Honig, and Metzler (2001), Zuo et al. (2008), and Mantovani et al., (2009), however, in this study ethanol was used as extraction solvent. The pharmacological potential of *C. ternatea* L. and *O. viciifolia* Scop. May be due to the presence of a broad range of secondary metabolites such phenolic compounds, anthraquinones, steroids, stilbenoids and piperidine alkaloids. The secondary metabolites include phenolic compounds, saponins and non-protein amino and imino acids, and an over expression of protein amino acids, and galloyl tyrosine (Alvarez et al., 1998; Lokvam et al., 2007).

Previous study on antimicrobial activity on Leguminosae was reported by Daniel et al. (2014) regarding methanol extract from roots of *Tephrosia toxicaria* inhibited the growth of *Bacillus subtilis* (8 mm), whilst Ethyl acetate extract from leaves of *Cassia leptophylla* inhibited also the growth of *Bacillus subtilis* (8 mm). Daniel et al. (2014) observed the antimicrobial activity from ethanolic extract from leaves of four Leguminosae (*Pimenta pseudocaryophyllus, Erythrina speciose, Inga marginata and Cassia leptophylla*), which concluded the inhibition zones; *Cassia leptophylla* (8 mm against *Bacillus subtilis*), *Erythrina speciose* (9 mm against *Candida albicans*), *Pimenta pseudocaryophyllus* (11 mm against *Candida tropicalis*), *Erythrina speciose* (11 mm against *Candida tropicallis*) and *Inga marginata* (9 mm against *Candida tropicallis*). According to Vasconcellos (2014), soybeans (legumes) exhibited stronger antimicrobial activity against tested bacteria (*Staphylococcus aureus* and *Pseudomonas aeruginosa*), with the exception of *E. coli.*

Previous study found the seeds of legumes are particularly rich in lectins, and many of these lectins have been characterized extensively (Goldstein and Hayes 1978; Lis and Sharon 1986). Legume lectins are proteins or glycoproteins of a ubiquitous distribution in nature, which have at least one carbohydrate or derivative binding site without catalytic function or immunological characteristics. Complete amino acid sequences of Concanavalin A (Edelman et al. 1972), favin (Cunningham et al., 1979), and lectins from lentil (Foriers et al., 1981), sainfoin (Kouchalakos et al., 1984), Phaseolus vulgaris (Hoffman et al., 1982), soybean (Hemperly et al., 1983), and pea (Higgins et al., 1983) have been determined. It is clear that these lectins have been conserved during evolution of the legumes and that the homologies in their NH_2 terminal amino acid sequences reflect the taxonomical relationships of the plants in this family (Foriers et al., 1977; 1979). Charungchitrak et al.(2011) reported *Archidendron jiringa* seed lectin was selected to test for antimicrobial activity with *Escherichia coli, Pseudomonas auroginosa, Bacillus subtilis, Staphylococcus aurous* and *Candida albican*.

Another interesting feature about *C. ternatea* L was the natural colourant from vivid blue colured petals which commonly used as natural food colorant or dye. However, for *O. viciifolia* Scop. in the temperate countries, usually grown as forage crop and the flowers are collected as honey product. Unfortunately, the present study only focused on *C. ternatea* L. flowers as natural colorant for coating technology, since *O. viciifolia* Scop. did not produce any flower in the current investigation in Malaysia. Petals of *C. ternatea* L. or commonly known as 'Bunga Talang' are consumed only to make a famous local dish, 'Nasi Kerabu', which is blue in colour (natural food dye). Therefore, the present study also focused on anthocyanin stability from *C. ternatea* L. petals as natural colourant for coating material, which is another aspect of application (Chapter 9). Anthocyanin content has a critical role in the colour quality of coloured flowers (Rajendran, 2010) and

fruits (Lohachoompol et al., 2004). Moreover, anthocyanin beneficial in reduction of coronary heart disease (Bridle and Timberlake, 1996), antioxidant (Takamura and Yamagami, 1994; Wang et al., 1997), anticancer (Karaivanova et al., 1990; Kamei et al., 1995) and improved visual acuity (Timberlake and Henry, 1988). Fukumoto and Mazza (2000) concluded that antioxidant activity usually increased with addition of anthocyanin. According to Parisa et al. (2007), the stability of anthocyanin pigment is depends on various factors including structure and concentration of anthocyanin, pH, temperature and presence of complexing agent. Thus, in the present study focused on the effects of pH, UV radiation, NaCl concentrations and heating duration on anthocyanin extracted from *C.ternatea* L. flowers.

In the present study, under normal light condition (fluorescent lamp) at room temperature, the colour of *C. ternatea* L. changed upon the exposure at different pH. The extract from vivid blue coloured of *C. ternatea* L. petals became red at pH 1, purple at pH 4.5, blue at pH 5.5 and cyan at pH 6.5 (Figure 9.2), maybe due to the change in the pigment's structure. Furthermore, this characteristic role as a strong indicator for presence of anthocyanins (the fact that the colour changed with pH) as compared to betalain (the same red pigment but do not change with pH), while, the flower extracted with water showed navy blue colour at pH 5.5. The best bright red colour extract was observed at pH 1 and pH 4.5. At pH 5.5 and pH 6.5, the red colour changed to green, while at pH 10 and pH 12, the colour changed to brown. The graph in Figure 9.3 showed absorbance values for pH 1, pH 4.5 and pH 5.5. The maximum absorbance (0.6048) was obtained at pH 1 and the minimum (0.4164) at pH 5.5, which was irradiated at 550 nm of wavelength, which is apparent for anthocyanins. Overall, based on the absorption spectra (the fluorescence intensity of the sensor increased with the increase in pH value), the anthocyanins were stable under strongly acidic (pH 1), but unstable in weakly acidic condition (pH 4.5 and

5.5). Therefore, the colour faded faster at higher pH (pH 1) than lower pH (pH 4.5 and 5.5). Vivid blue petal flowers of *c. ternatea* L. contain mainly ternatins (polyacylated delphinidin 3,3',5'-triglucosides) and preternatins , which change the flower colour due to lack of glucosyl subsitutions at both 3'- and 5'- positions of the ternatins (Kazuma et al., 2003).

The graph in Figure 9.4 showed absorbance values for UV radiation within 0, 60 and 120 min. Conventionally, UV radiation play an important role in killing microorganisms in food and beverages. In the present study, the results obtained the maximum absorbance (0.6048) in 0 min at 550 nm, whilst the minimum (0.4164) irradiated at 600 nm of wavelength at pH 1. Overall, the absorbance values decreased in higher duration of UV radiation exposure at a faster rate. Moreover, the original red colour was remaining the same after 24 hours. However, according to Tantituvanont et al. (2008), the anthocyanin extracted from *C. ternatea* was preserved better when kept in the dark than exposed to UV light, which the percentage colour remaining increased approximately 20% from its original absorbance and maintained at this value until day 60. Roobha et al. (2011) reported that the anthocyanin extract was stable whether in presence or absence of light, in condition that the pH range was at 5.1-6.0 and temperature was at 20-30ºC).

Generally, anthocyanins can undergo reversible structural change according to different pH condition. There are four major anthocyanin forms that exist in equilibrium; the red flavylium cation, blue quinonoidal base, colourless carbinol pseudobase and colourless chalcone.Kohno et al. (2009) claims that the increasing pH values (between pH2 and pH 4) could cause red flavylium cation to deprotonate easily to form the blue quinonoidal base, whilst when the pH increase further, occurs hydration of flavylium cation, generating the carbinol pseudobase which can undergo ring opening to form chalcone. The graph in Figure 9.5 showed absorbance values for different NaCl concentrations (0, 20 and 30g/L). The

results obtained the maximum absorbance (0.6048) in 0 g/L NaCl and minimum (0.4999) in 30 g/L, which was irradiated at 550 nm of wavelength at pH 1. Overall, the absorbance values decreased in higher NaCl concentrations with the same red colour (as original) after 24 hours. At pH 1 (the strongest acidic condition), the anthocyanin exist primarily in the form of flavylium cation in red, which the most stable and bright in colour (Kohno et al., 2009). Moreover, in pH values between 4.0-5.5, the colourless carbinol and yellowish chalcone dominate, whereas the carbinol form has lost its conjugated double bond between A- and B-ring and therefore does not absorb the visible light (Rein, 2005).

Originally, the heating test aims to investigate the effects of hot water or weather to the stability and durability of anthocyanin (natural colorant) such as in production and storage of baby products and paint. During the heating test, the red colour (at pH 1) of this anthocyanin extraction became darker in the oven (50±1ºC) as the duration of heat increased. This characteristic (the colour became darker, instead of fade), which beneficial as a stable natural colourant as shown in Figure 9.6, the absorbance values for different heating duration (0, 20 and 30 min). The results obtained the maximum absorbance (0.6048) in 10 min at 550 nm, whilst the minimum (0.4323) in 30 min irradiated at 600 nm of wavelength at pH 1. Overall, the absorbance values decreased in higher duration of heating in the oven, with the same red colour (as original) after 24 hours. Turkey et al. (2004) stated that the highest anthocyanin retention is observed at 4ºC of storage temperature compared to 25ºC and 40ºC. Goto-Yamamoto reported that high temperature (30-35ºC) significantly reduced the anthocyanin concentration, particularly on the structure of cyanidin and peonidin-3-glucosides of grape berry skin. Moreover, combining pressure to the heat treatment resulted in slightly faster degradation of anthocyanins from blueberry (Buckow et al., (2010) and blackberry (Wang and Xu, 2007).

Anthocyanins extracted from crude petals of *C. ternatea* L. was easily oxidized when exposed to the room temperature. The best bright red colour extract was observed at pH 1. Therefore, in the aim of retaining the bright red color, the anthocyanin extract was mixed with 20% polymethylmetacrylate (PMMA) as co-pigmentation effect of organic acids (tartaric and citric acid) at pH 2 were coated onto glass slides (Figure 9.7). Initially, the anthocyanin colorant, the best bright red colour extract obtained by using methanol acidified with 0.5% trifluoroacetic acid (TFA).

The durability of coating was observed under different stability with addition of organic acid which all the absorbance values were decreased within 8 days (Figure 9.8). Tartaric acid was found to be the best stabilizer for anthocyanin to improve the colourant stability of *C. ternatea* L. The complexes formed between anthocyanin-PMMA and tartaric acid known as copigmentation. According to Christine and John (1995), various natural chemicals can act as copigment including organic acids, amino acids, alkaloids, polyphenols and flavonoid. The tartaric acid added colourant was mixed with PMMA and coated onto glass slides to develop a coating system. In the liquid form, the anthocyanin-PMMA mixture added with tartaric acid showed the highest absorbance, which explained that tartaric acid can enhance the intensity of absorbance of the liquid coating mixture. The anthocyanin-PMMA mixture added with citric acid showed the lowest absorbance as compared to the mixture containing tartaric acid or without any organic acid (control). Thus, coating with 1% tartaric acid showed the best enhancement and stability (the colour remain as same as original, but without degradation), as the copigment enhances the colour of extracted solution. Copigmentation between anthocyanins and copigments isone of the significant factors of structure stabilization under *in vivo* condition (Marcovic et al., 2000). Agreed with this, Abyaniet al. (2006) and Awika (2008) repoted that cinnamic acid could form stable complexes with anthocyanins.

Vivid blue flower of *C. ternatea* L. contains flavonoid such as quercetin, kaemferol, robinin and clitorin, as well as several glycosides including malvidin-3-β-glycosides, dephinidin-3-β-glycoside (Srivastava and Pandey, 1977) and dietary anthocyanins (Terahara et al., 1996). The anthocyanins are responsible for the water soluble, vacuolar, pink, red, purple and blue pigments present in coloured plant pigments. According to Cunningham and Gantt (1998), these pigments are important agronomic value in many crops and ornamental plants. However, anthocyanins are not stable and easily to degrade and fade whenever expose to the light. Due to natural colorant safety and health benefit, as well as strong consumer demand for more natural products (Wong, 2008), *C. ternatea* L is used for ornamental, as well as for dye and ethno-medicine (Cook et al., 2005).

The anthocyanin extract of *C. ternatea* L. contains anxiolytic, antidepressant, anticonvulsant and antistress properties (Jain et al., 2003). According to the traditional system of medicine 'Aparajita' is considered as a 'Medhya' drug to improve intelligence and enhance memory function (Kulkani et al., 1988). It is also used in the treatment of chronic bronchitis, dropsy, goiter, leprosy, mucous disorders, sight weakness, skin diseases, sore throat and tumors. In Ayurveda Indian medicine, the roots are most widely used and are bitter, refrigerant, laxative, intellect promoting, diuretic, anthelmintic and as tonic. This root is useful in dementia, burning sensation, inflammation and asthma. The seeds are cathartic, while the leaves are used in otalgia and hepatopathy. Besides, the roots, stems and flowers are recommended for the treatment of snakebite and scorpion- sting. These may be due to photochemical constituents like flavonoid, alkaloids and tannins of plants (Bose et al., 2007).

Colorants are often added in food to enhance its visual aesthetics and to promote sales (Huang et al., 2002). Although the amount of synthetic colorants is reduced for consumer health reasons in recent years, many kinds of synthetic food

dyes are still widely used all over the world due to their low price, high effectiveness and excellent stability. Generally, synthetic colorants can be classified into water-soluble and fat-soluble colorants based on their solubility. Most fat-soluble synthetic colorants present in the market are hazardous compounds, such as Sudan I, Sudan II, Sudan III, and Sudan IV. The genetic toxicity of some azo-dyes has been confirmed (IARC, 1975; Calbiani et al., 2004) and structure–activity relationships have been assessed (Searly, 1976; Prival et al., 1988). It is well known that Sudans (I–IV) have been classified as category 3 carcinogen to humans by International Agency for Research on Cancer (Tateo and Bononi, 2004), and the use of Sudan I in foodstuff is forbidden in global food regulation act (DiDonna et al., 2004). However, Sudan dyes were still found in food products exported in European countries (Calbiani et al., 2004). Therefore, in this study, the extracts of *C. ternatea* L. Flowers could be potential as natural colorant with no harm to mankind.

Anthocyanin extract is a natural plant pigment which is non-toxic (free from volatile organic components) and environmental friendly. Anthocyanins and flavonols are two major subclasses of flavonoid compounds existing widely in flowers, fruits and vegetables. According to Dugas et al.(2000), quercetin was the best scavenger of peroxyl radical out of seven common flavonoids. In view of the mass consumption of medicinal and functional food, it is of great importance to focus on *C. ternatea* L. in these active components for further investigation. This anthocyanin is suitable as natural colorant especially in baby products or cosmetics production or for coating and varnish application. The combination of binders and pigments produced environmental paint which added with stabilizers (additives) for better durability (Abidin et al, 2006).

Anthocyanins are responsible for the water soluble, vacuolar, pink, red, purple and blue pigments present in coloured plant pigments (Lewis et al., 1998; Mancinelli, 1985). Anthocyanins have been used as coloring for food and

beverages; however, many anthocyanins are unstable in neutral solutions and lose their color, thus their use for food and pharmaceutical products, among others, has been limited (Dougall and Baker, 2008). The reasons for increasing the use of these colorants could be justified by their beneficial health effects (Torskangerpoll and Andersen, 2005). Anthocyanins have a useful potential as natural colorants due to their attractive colors (Markakis, 1982); however, their usage in the food industry is limited due to their instability when exposed to factors like environmental variations, including temperature, light intensity, oxygen (Delgado-Vargas et al., 2000) and pH (Fossen et al., 1998).

Regardless of the type of colorants, the pigments are incorporated either under their natural occurrence or under a chemical modified form. As a consequence of these additional pigment needs, the demand in isolated natural colorants has increased as compared with synthetic dyes (Pszczola, 1998). However, this need cannot always be satisfied due to the limitation in the supply of raw materials because the production of pigments using conventional plant cultivation methods is influenced by climatic conditions, plant cultivars and varieties (Rodriguez-Amaya, 2000). Thus, with the advantages of Malaysian climate, temperatures and soil for vigorous and mass cultivation of *C. ternatea* L., all year long, especially is using *in vitro* technique which could be beneficial as source of natural colorant, especially in paint production, as well as for honey production (extend to honey production from *O. viciifolia* Scop. in temperate regions). Therefore, the stability of anthocyanin-based natural colourants with organic stabilizer and antimicrobial properties could be very beneficial to protect against allergy ordiseases, besides for food, beverages, paint, varnish and cosmetics.

CHAPTER 11

CONCLUSION

The present study revealed the potential of *Clitoria ternatea* L. (tropical legume) and *Onobrychis viciifolia* Scop. (temperate legume) to be introduced as multi-purpose legume plants in Malaysia and to be cultivated at larger scale. Initially, the germination was observed under different temperatures (18, 24 and 30±1ºC) to determine the specific capability of these species to respond to high and low temperatures, both *in vivo* and *in vitro*. The best temperature for seed germination for *C. ternatea* L. and *O. viciifolia* Scop. was at 24±1ºC, grown *in vivo* and *in vitro*. Subsequently, through tissue culture system (aseptic conditions at 24±1ºC), seeds were germinated on MS basal medium. Unfortunately, even though the aseptic seedlings grew vigorously and healthy, however, no regeneration was obtained from root and stem explants (1-month-old) of *C. ternatea* L., while, *O. viciifolia* Scop. had a problem in producing healthy normal leaves, though with a lot of stems. After 6 months observation on the seeds germination, a structure of white callus was formed at the roots and stem of *C. ternatea* L., which promoted shoots and roots induction. Therefore, different concentration of $ADSO_4$ (0-100 mg/L) were added to the MS basal media to enhance the white callus structure in shorter time. Fortunately, within 10 days, both species formed callus at root explants on MS basal media supplemented with 40 mg/L $ADSO_4$. Based on this result ($ADSO_4$-added MS media as control), root, stem and flower bud (obtained from *C. ternatea* only) explants were cultured on MS medium supplemented with 40 mg/L $ADSO_4$ and various plant hormones (NAA, BAP, 2,4-D and KIN) at concentrations of 1.0-3.0 mg/L, applied singly and in combinations. In week 2, all explants produced callus, but root explant was identified as the most responsive to

form shoots and roots for both species. The optimum media for *C. ternatea* L. was MS supplemented with combinations of 40.0 mg/L $ADSO_4$, 2.0 mg/L NAA and 1.0 mg/L BAP with shoots (12.03±0.12) and roots (8.72±1.27) formation. The optimum media for *O. viciifolia* Scop. was MS supplemented with combinations of 40.0 mg/L $ADSO_4$, 1.0 mg/L 2,4-D and 3.0 mg/L KIN with shoots (17.97±0.09) and roots (4.11±0.42) formation. Generally, *C. ternatea* L. was more responsive to $ADSO_4$-added MS media supplemented with NAA and BAP, compared with *O. viciifolia* Scop. which preferred $ADSO_4$-added MS media supplemented with 2,4-D and KIN. *O. viciifolia* Scop. grew two times faster than *C. ternatea* L. The present study was also focused on callus production from 31 treatments with manipulation and exploitation of $ADSO_4$ (0-100 mg/L), auxins (NAA and 2,4-D, 1.0-3.0 mg/L), cytokinins (BAP and KIN, 1.0-3.0 mg/L) and explants (root, stem and flower bud) using MS basal media, which resulted in all explants produced callus within a week. The embryogenic (stained intense bright red with acetocarmine) and non-embryogenic (stained with Evan's blue) callus was distinguished by double staining method. This callus have been used as somatic embryo encapsulated in 3% sodium alginate for synthetic seeds production as another alternative for germination and regeneration (without using natural seeds).

Due to limitation of seeds, especially for imported species (*O. viciifolia* Scop.), synthetic seeds production was another alternative method for mass propagation which can be stored under low temperature up to six months. Thus, in subsequent work, the optimum concentration for the formation of encapsulation matrix was 3.0% (w/v) sodium alginate ($NaC_6H_7O_6$) soaked in 100 mM (w/v) calcium chloride dehydrate ($CaCl_2.2H_2O$) solution for 30 minutes. For three months, the synthetic seeds of *C. ternatea* L. and *O. viciifolia* Scop. still viable for germination with 61.7±0.9% and19.2±0.3% germination rate, respectively. The

embryogenic callus formed through four main different stages (globular, heart, torpedo and cotyledonary) with the highest percentage from root explants of *C.ternatea* L. (0.31±0.02 g) cultured on MS medium supplemented with 40.0 mg/L $ADSO_4$, 2.0 mg/L NAA and 1.0 mg/L BAP and for *O. viciifolia* Scop (1.44±0.02 g) on MS medium supplemented with 40.0 mg/L $ADSO_4$, 1.0 mg/L 2,4-D and 3.0 mg/L KIN. After 20 weeks, the embryogenic callus formed into shoots and roots as complete plantlets ready for acclimatization. In this study, the focus was more on regenerated plants of *C. ternatea* L. and *O. viciifolia* Scop. from root explants, after 7 months under *in vitro* condition. The highest survival rate (91.07±0.96%) obtained from plantlets of *C. ternatea* L., on mud soil (pH 4.26±0.12), while, *O. viciifolia* Scop., (71.58±1.27%) on top soil (pH 5.14±0.12).

After 7 months being acclimatized, the *ex vitro* plants of *C. ternatea* L. was shorter in height (39.16±0.28 cm), which was about half of the *in vivo* plant height (95.03±0.83), but the quality of leaves (2.11±0.11 cm) and flowers (9-16 flowers per plant) were better, and good nodules formation (25-40 nodules per plant). Hence, the examination of the *ex vitro* plant was compared to *in vitro* plants on micromorphological distribution of stomata and trichomes on the leaves of both species, using Field Emission Scanning Electron Microscope (FESEM) and histology analysis. The FESEM analysis showed the presence of non-glandular trichomes with sharp-point end. Histologically, the adaxial (upper epidermis) from *in vivo* grown leaf of both species showed the presence of cuticle which affected by the sunlight exposure, besides providing protection against desiccation and pathogens. Overall, the ultrastuctural features of *in vivo* leaf showed more air space in palisade and spongy mesophyll layer, while the *in vitro* leaf with compact cells. After 6 months, the regenerated plants showed almost similar growth characteristics as the mother plants (with same morphology, histology, anatomy and ultrastructural

features), implying that no somaclonal variation occurred during the *in vitro* growth of both species. As known worldwide, all plants produced their own phytochemical substances, especially to protect against some particular microorganisms. Therefore, through tissue culture system, callus and leaves from regenerated plants derived from root explants were tested for antimicrobial activity and compared to the *in vivo* plants of the same age. The results of antibacterial activity validated the highest inhibition zone of *C. ternatea* L. from *in vitro* leaves extracts (15.00±0.56 mm) which was comparable to *in vivo* plants (16.00± 0.35 mm) against *Bacillus subtilis, w*hilst, *in vitro* leaves extraction of *O. viciifolia* Scop. responded differently (against *Staphylococcus aureus*, 12.00±0.39 mm) as compared to *in vivo* plants (against *Escherichia coli*, 17.00±0.63 mm). Surprisingly, both callus extraction of *C. ternatea* L. (15.00±0.37 mm) and *O. viciifolia* Scop. were found to respond against *Escherichia coli.* For antifungal activity, both callus extract (14.00±0.46 mm) and *in vitro* leaves extract (13.00±0.32 mm) of *C. ternatea* L. gave the best response against *Fusarium* sp. as compared to *in vivo* leaves extract (11.00±0.35). In addition, *in vitro* leaves extraction (13.00±0.57 mm) was comparable to *in vivo* leaves extraction (15.00±0.55 mm) against *Candida albican*. However, antifungal activity of *O. viciifolia* Scop. From *in vivo* plants (18 mm) was better compared to *in vitro* extraction (11-16 mm) against *Fusarium* sp and *Trichoderma* sp.

In Malaysia, flowers of *C. ternatea* L. are only used in making 'Blue Nasi Kerabu' preparation as blue dye, while, flowers of *O. viciifolia* Scop. in temperate regions are collected as honey product. Since, the flowers of *C. ternatea* L. is easier to get in Malaysia, therefore, the subsequent study was focused on the extraction from vivid blue coloured petals. Thus, the identity and stability of anthocyanin extract was determined through pH variation test, UV exposure and NaCl using UV-visible spectrophotometry. In addition, this anthocyanin was subjected also to antimicrobial activity and compared with non-embryogenic callus that was

derived from flower buds cultured on MS+40.0 mg/L $ADSO_4$+3.0 mg/L 2,4-D. Generally, 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 (*Bacillus subtilis*) with 11 mm and 10 mm inhibition zones, respectively. However, different antifungal activity was detected from *in vitro* ethanolic callus extract (12 mm) which was shown against *Trichoderma* sp., contrary to *in vivo* ethanolic extract (10 mm) which was against *Fusarium* sp.

Generally, *C. ternatea* L. capable to adapt in both tropical and temperate regions due to the ability for temperature tolerance, vigorous growth with high biomass production, drought resistance, shade tolerance, non-palatibility, as well as pest and disease tolerance. The most important fact, *C. ternatea* L. and *O. viciifolia* Scop. are Leguminosae that are beneficial for organic farming (can fix nitrogen from the air) and as green technology for sustainable environment. In addition, through tissue culture technique, both species can be supplied year round for commercial cultivation without any geographical and seasonal constraints. For future work, cytological studies of *C. ternatea L* (local tropical legume) and *O. viciifolia Scop.* (imported temperate legume) need to be investigated to understand the cellular behaviour of these plants. The studies on Mitotic Index, ploidy level, chromosome number, cell cycle, nuclear and cell areas can determine variations of the plant genotype and phenotype, grown *in vivo* and *in vitro*, as well as under different environmental conditions. Molecular aspects of these species is also lacking, hence, further work and investigation are needed for these important leguminous forage crops to be commercialised and exploited fully.