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±1°C), adenine hemisulfate (ADSO₄, 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±1°C, 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±1°C) 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 25°C, whilst 63% germination rate at 20°C on half strength MS medium. Similar with this, Rojas-Árêchiga 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±1°C. However, the growth development of *O. viciifolia* Scop. was two times faster than *C. ternatea* L. when grown at 18±1°C. 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 ADSO4, 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 ADSO4, 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 ADSO4, 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 ADSO4-added MS media supplemented with NAA and BAP, while O. viciifolia Scop. Performed best on ADSO4-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 ADSO4 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 ADSO4, 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 ADSO4, 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 ADSO4 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 ADSO4, 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 ADSO₄-added MS media supplemented with NAA and BAP, while *O. viciifolia* Scop. was more suitable to be cultured on ADSO₄-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 ADSO₄ and 2.0 mg/L KIN), followed by stem explant (0.23±0.01 g, cultured on MS supplemented with 40.0 mg/L ADSO₄ and 3.0 mg/L KIN) and flower bud explant (0.21±0.05 g, cultured on MS supplemented with 40.0 mg/L ADSO₄, 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 ADSO₄, 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 ADSO₄, 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 ADSO₄ and 3.0 mg/L 2,4-D with 0.18±0.07 g 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 ADSO₄ 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 ADSO₄, 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 ADSO₄ 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 the perennial 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. Zăpărtan, 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 (6-7 shoots/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 ADSO4 (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 conjunction 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 ADSO4, 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 embryonic 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 ADSO4 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 ADSO4, as well as 2,4-D,
KIN and ADSO4. 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 (NaC6H7O6) and soaked in calcium 100
mM (w/v) chloride dehydrate (CaCl2.2H2O) 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±1°C) 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 vicifolia* Scop. (Figure 6.2) died after 2 months being exposed to Malaysian temperature (30±1°C) 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 adaptation 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 N2 to NH3) 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 alfalfa, 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 greenhouse.

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°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±1°C for adaptation or acclimatization process. Then, the plantlets were transferred to the greenhouse at 30±1°C. The macromorphological 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±1°C). 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 are required 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 subsidiary 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 occurred 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 \textit{in vitro} culture plant also has slightly thick leaf as compared to \textit{in vivo} culture plant. Furthermore, \textit{in vitro} culture plant has less wax compared to intact plants. This might be because of \textit{in vitro} culture plant was cultured under sterile conditions and the wax layers may not developed well as in \textit{in vivo} plants.

The study on leaf gross morphology and structural features under FESEM showed that \textit{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 ADSo4 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 25-30°C 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 \textit{in vivo} plants. Antimicrobial activity differs in \textit{in vivo} and \textit{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 \textit{C. ternatea} L. and \textit{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 \textit{in vitro} antimicrobial properties (Gonzalez-Lamothe et al., 2009). Thus, based on the results in Chapter 8, \textit{C. ternatea} L. and \textit{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 \textit{in vivo} leaves extraction (mother plant) of \textit{C. ternatea} L. against both \textit{Bacillus subtilis} and \textit{Escherichia coli}. In single function, \textit{in vitro} leaves extraction can be proposed as anti-\textit{bacillus subtilis} (15.00±0.56 mm), while callus extraction as anti-\textit{Escherichia coli} (15.00±0.37 mm). The highest inhibition zone (17.00±0.65 mm) was from \textit{in vivo} leaves extraction of \textit{O. viciifolia} Scop. against \textit{Escherichia coli}. While, the ability of callus extraction (15.00±0.47 mm) was comparable to mother plants (16.00±0.61 mm) against \textit{Staphylococcus aureus}. 

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The highest inhibition zone (15.00±0.55 mm) of antifungal activity of \textit{C. ternatea} L. obtained from \textit{in vivo} leaves extraction against \textit{Candida albicans}, as well as by \textit{in vitro} leaves extraction (13.00±0.57 mm). Furthermore, comparing among \textit{in vivo} and \textit{in vitro} leaves extraction, callus performed the best in inhibition of \textit{Fusarium} sp. (14.00±0.46 mm). All extractions of \textit{O. viciifolia} Scop. (\textit{in vivo} leaves, \textit{in vitro} leaves and callus), gave positive response as antifungal activity to both \textit{Fusarium} sp. and \textit{Candida albicans}. However, the highest inhibition zone (18 mm) by \textit{in vivo} leaves extraction, followed by callus extraction (15-16 mm) and \textit{in vitro} leaves extraction (11.00 mm). Both species (\textit{C. ternatea} L. and \textit{O. viciifolia} Scop.), responded negatively to antifungal activity against \textit{Trichoderma} sp. However, \textit{O. viciifolia} Scop. Was detected as anti-\textit{Fusarium} sp. and anti-\textit{Candida albicans}. Generally, the present results verified that the presence of antimicrobial activity against bacteria (\textit{Bacillus subtilis}, \textit{Staphylococcus aureus} and \textit{Escherichia coli}) and fungi (\textit{Fusarium} sp., \textit{Candida albicans} and \textit{Trichoderma} sp.) varied with different explants source (callus and leaves) of both species, grown \textit{in vivo} and \textit{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 \textit{Clitoria ternatea} L., which successfully observed the antibacterial activity (both \textit{in vivo} and \textit{in vitro} extracts against \textit{Bacillus subtilis}), however different antifungal activity was detected,
whereby the \textit{in vitro} extract was against \textit{Trichoderma} sp., contrary to \textit{in vivo} extract that was against \textit{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 \textit{C. ternatea} L. and \textit{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 \textit{Tephrosia toxicaria} inhibited the growth of \textit{Bacillus subtilis} (8 mm), whilst Ethyl acetate extract from leaves of \textit{Cassia leptophylla} inhibited also the growth of \textit{Bacillus subtilis} (8 mm). Daniel et al. (2014) observed the antimicrobial activity from ethanolic extract from leaves of four Leguminosae (\textit{Pimenta pseudocaryophyllus, Erythrina speciose, Inga marginata} and \textit{Cassia leptophylla}), which concluded the inhibition zones; \textit{Cassia leptophylla} (8 mm against \textit{Bacillus subtilis}), \textit{Erythrina speciose} (9 mm against \textit{Candida albicans}), \textit{Pimenta pseudocaryophyllus} (11 mm against \textit{Candida tropicalis}), \textit{Erythrina speciose} (11 mm against \textit{Candida tropicalis}) and \textit{Inga marginata} (9 mm against \textit{Candida tropicalis}). According to Vasconcellos (2014), soybeans (legumes) exhibited stronger antimicrobial activity against tested bacteria (\textit{Staphylococcus aureus} and \textit{Pseudomonas aeruginosa}), with the exception of \textit{E. coli}. 

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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 NH2 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 colored 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 20-30°C.

Generally, anthocyanins can undergo reversible structural change according to different pH condition. There are four major anthocyanin forms that exist in equilibrium; the red flavlyium cation, blue quinonoidal base, colourless carbinol pseudobase and colourless chalcone. Kohno et al. (2009) claims that the increasing pH values (between pH 2 and pH 4) could cause red flavlyium cation to deprotonate easily to form the blue quinonoidal base, whilst when the pH increase further, occurs hydration of flavlyium 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 is one of the significant factors of structure stabilization under *in vivo* condition (Marcovic et al., 2000). Agreed with this, Abyaniet al. (2006) and Awika (2008) reported 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, dephdinidin-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, refrigerator, 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 peroxy 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.