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 (49°C) for overnight and transferred into melted paraffin wax (56°C) for 24 hours. Embedding process was done by pouring 56°C 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™ 450, EDX-OXFORD FE-SEM) and Axio vision 4.7 software (Axioskop Zeiss GERMANY microscope).
7.3 RESULTS

7.3.1 Comparison 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 vicifolia* 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 vicifolia* 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 vicifolia* 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.