Chapter 3: Materials and Methods

3.1 MATERIALS

Herbarium specimens of *Alternanthera* from several herbaria (KLU, KEP, UKMB and SING) were checked before and after field trips. The specimens were checked and information such as locality, morphology, habitat, date of collection and the name of collector were recorded. Several field trips were carried out in Peninsular Malaysia to collect specimens (Appendix 3.1). Specimens were collected from different localities to prevent biased results from dealing with a single genotype. Three species were found during the field trips which are *A. sessilis* 'Green', *A. paronychioides* and *A. ficoidea*. *Alternanthera brasiliana*, *A. sessilis* (red) and *A. bettzickiana* are all cultivated species and have not been seen growing wild in the field. The voucher specimens of *Alternanthera* species studied were deposited at the Herbarium Institute of Biological Sciences, University of Malaya (KLU). Location of study site and the collection numbers of the respective species are listed in Appendix 3.2.

3.2 STUDY SITES

Specimens of *Alternanthera sessilis* (green) and *A. bettzickiana* were collected from the field and then planted outside the green house at the Institute of Biological Sciences, Faculty of Science, University of Malaya. The study site of *A. sessilis* 'Red' was located at the Rimba Ilmu Botanic Garden, University of Malaya. Both red and green leaf forms of *A. sessilis* were closely monitored from June 2009 to May 2010. Specimens of *Alternanthera bettzickiana* were closely monitored from October 2010 to March 2011.

The study site of the parent plant of *A. brasiliana* was located near the car park of the Institute of Biological Sciences, Faculty of Science, University of Malaya. The specimens were closely monitored during the flowering period, which was from

November 2009 to March 2010. The seeds collected from the parent plants were germinated and planted outside the green house at the Institute of Biological Sciences, Faculty of Science, University of Malaya. Similar to the parent plants, the offspring were closely monitored during the flowering period, which was from October 2010 to April 2011.

3.3 METHODS

A dissecting microscope model Leica MZ9.5 and binocular microscope model Leica DM2500 attached with a digital camera (Leica DFC420) were used for this study. In the field, measurements were taken using a digital vernier caliper (Mitutoyo 500-181-21) whereas observations were carried out using a hand lens (magnification 10x) and headlamp.

For the statistical analysis, the distribution pattern of entire variables were tested by Kolmogorov-Smirnov and a one-way ANOVA. If the one-way ANOVA test showed a significant result, a Tukey's post hoc test or Games Howell post hoc test would be computed. An independent sample t-test or Mann-Whitney U test for entire variables would be conducted if the homogeneity assumption of ANOVA had been violated. The analysis was performed using SPSS 11.5 for Windows.

The materials required for the different aspects of studies were collected and treated in the following manner.

3.3.1 Plant morphology

Three individual plants of *A. sessilis* (both green and red), *A. paronychioides* and *A. bettzickiana, A. ficoidea and A. brasiliana* from different localities (Appendix 3.1) were randomly selected for the morphological study. Mature flowers, foliage and stems from each plant were collected and observed under the dissecting microscope. Both

qualitative and quantitative data were recorded. The qualitative data included the morphology of the foliage, stem, flower, androecium and gynoecium. The quantitative data included the measurement of the length and width of the leaf, bract, bracteoles and petal. The length of the stamen, ovary and style was recorded as well. The terminology used generally followed that of Harris & Harris (1997).

3.3.2 Chronology and sequence of development of flower and fruit

A. Inflorescence development and longevity

The inflorescences were randomly tagged when they were visible. In order to avoid inflorescence length differences between samples, inflorescences with similar lengths were used. Hence, the length of the inflorescence was about 1.5 mm in *A. sessilis* (both red and green), 3.0 mm in *A. brasiliana* and 3.5 mm in *A. bettzickiana*. The length of the inflorescences as well as the number of days taken for the inflorescences to mature (when the first flower on an inflorescence bloomed) and reach full bloom was recorded. The sequence of flower development and number of flowers reaching anthesis in an inflorescence were also recorded.

B. Flower anthesis

In *A. sessilis* and *A. bettzickiana*, flower anthesis was monitored from 0700–1800 hours. The time of flower anthesis, anther dehiscence and flower closing were recorded. After closing, the mature flower was collected and its length was measured. Dehiscence began when the longitudinal slits on an anther broke open and was considered complete when all the anthers in a flower had opened and released their pollen grains. Dehiscence was deemed incomplete as long as one or more anthers had not dehisced.

In *A. brasiliana*, flower anthesis was monitored in the parent plant from 0600–2330 hours. The time of anthesis and flower closing was recorded. The flower anthesis process was divided into three stages: (1) Mature bud opened slightly, the stigma and anthers could not be seen as the opening was tiny; (2) Mature bud opened bigger, the stigma could be seen and (3) Mature bud opened until both the stigma and anthers could be seen. Although anther dehiscence did not occur in *A. brasiliana*, the morphology of anther and pseudostaminodes during flower anthesis was observed as well.

To study stigma receptivity of *A. sessilis* and *A. bettzickiana*, the stigmas were observed throughout flower anthesis. To study stigma receptivity of *A. brasiliana*, the stigmas were observed throughout flower anthesis (stage 2 and stage 3), one and two days after flower anthesis. These stigmas were collected once the flower started to bloom and when the flower was about to close. A drop of 3% hydrogen peroxide was then added to the stigma. A bubble seen released from the stigma indicated a positive result while the absence of a bubble indicated a negative result. Before the test was undertaken, the morphology as well as the size of the ovary was recorded.

C. Fruit development

To tag a single flower, the petals were marked with different colours using water resistant marker pens when it bloomed. For *A. sessilis*, fruits were collected on days 1, 3, 5, 7, 9, 11, 13 and 15 after flower anthesis. For *A. brasiliana*, fruits were collected on days 3, 5, 7, 9 and 11 after flower anthesis. The fruits were considered as mature when it could be detached easily without having to pull it from the inflorescence with forceps. The morphology and the size of the fruit, anther, pseudostaminodes and stigma of these flowers were examined. No fruits were produced in *A. bettzickiana*.

3.3.3 Embryological study

For the embryological study, the buds, flowers and fruits at different stages were collected and fixed immediately in Craf III solution. The species studied included *A. sessili*s 'Red' and 'Green', *A. brasiliana*, *A. ficoidea*, *A. bettzickiana* and *A. paronychioides*. Following standard procedures of microtechnique (Johansen, 1940) (Appendix 3.3), the specimens were embedded in Paraplast, sectioned and stained in safranin and fast green.

3.3.4 Palynological study

Anthers of 15 flowers of each species were collected just before flower anthesis or from the mature buds. The species studied included *A. sessili*s 'Red' and 'Green', *A. ficoidea* and *A. paronychioides*. The pollen grains were then acetolyzed according to the standard procedure of Erdtman (1960) (Appendix 3.4). The acetolyzed pollen samples were then divided into two; one for examination with the light microscope (LM) and the other for scanning electron microscope (SEM) study. Slides for LM were prepared with glycerine jelly stained with safranin and sealed with wax. 50 measurements of polar length (P) and equatorial diameter (E) were taken to determine the pollen shape and size as well as the ratio of P/E.

Pollen grains for SEM study were affixed onto carbon conductive adhesive tape and coated with gold at 20 mA for 90 seconds under the SPI-Module sputter coater. Specific characteristics of the aperture and sexine ornamentation were examined and photographed using the JOEL JSM-6400 scanning electron microscope.

The terminology used generally followed that of Faegri & Iversen (1950), Erdtman (1954), Borsch (1998) and Borsch & Barthlott (1998). Statistical analyses were used to compare the polar length, equatorial diameter, the diameter of pore, the height

of microspines and the number of ektexinous bodies attached on the pore membrane in each pore. In addition, the polar length and equatorial diameter of *A. sessilis* 'Red' and 'Green' from different habitats were also compared.

3.3.5 Pollen viability and germination test

In *A. sessilis* (both red and green leaf forms), preliminary tests on pollen germination were carried out using fresh pollen grains from 10 blooming flowers of 10 different plants. The pollen was cultured using the sitting drop technique (Shivanna & Rangaswamy, 1992) (Appendix 3.5) for eight hours at the temperature of 22°C in 10 different sucrose concentrations (4%, 6%, 8%, 10%, 12%, 14%, 16%, 18%, 20% and 22%) in Brewbaker and Kwack's medium (Brewbaker and Kwack, 1963) (Appendix 3.6). The cultures without any germination were left for another 24 hours before being checked again to confirm the germinability of the pollen in those media.

Pollen grains were considered to have germinated when the length of their tubes was equal or greater than the diameter of the pollen grains. The germinated pollen grains were fixed in 10% ethanol, stained with 0.1% neutral red and temporarily sealed with nail polish. The sucrose concentrations that showed favorable germination were used in subsequent replicates.

To analyze the percentage of germination, the total number of pollen grains and the number of germinated grains were recorded. The length of ten pollen tubes from each treatment was measured using a calibrated eyepiece micrometer under the binocular microscope. For the statistical analysis, the mean of pollen tube length from different concentrations was compared.

3.3.6. Pollination experiments

To investigate the nature of the breeding system in *A. sessilis* (both red and green leaf forms) and *A. brasiliana*, the following aspects were studied:

A. Pollen ovule ratio

Three matured flower buds from ten different plants were collected a day before they bloomed. The theca wall of an anther from each bud was spilt and mixed with a 5 µl detergent solution on a cavity slide. The total number of pollen grains in one anther was initially estimated by a haemocytometer. When this method proved to be ineffective, the pollen grains were counted under a binocular microscope using a simple telecounter. The pollen to ovule ratio was then determined following Cruden (1977).

B. Pollination experiments

Listed below are the pollination experiments that were performed (Dafni, 1992):

(a) Open pollination

The tagged inflorescences were left unbagged, undisturbed and opened to free pollination.

(b) Bagging (self pollination)

Two types of experiments were carried out. In the first experiment, an individual flower (from an inflorescence) which was about to reach anthesis was bagged. In the second experiment, the whole inflorescence was bagged and left untreated a day before the first flower reach anthesis.

(c) Geitonogamy

An individual flower (from an inflorescence) that has just begun to reach anthesis was hand-pollinated by pollen grains from a different flower in the same inflorescence before bagging.

(d) Emasculation and bagged (apomixis)

Individual emasculated flower from an inflorescence was bagged to ensure that it remained pollen-free.

(e) Cross pollination

A. sessilis 'Red' was hand-pollinated with pollen grains from *A. sessilis* 'Green' and bagged.

Prior to the bagging, emasculation and cross pollination experiments, all open or immature flowers were removed from the inflorescences using a pair of fine forceps. The flowers used in cross pollination and emasculation experiments were emasculated prior to anther dehiscence and bagged immediately after the treatments were conducted. The flowers were not emasculated prior to flower anthesis because the flower buds were too small and were vulnerable to injuries as a result of handling. The inflorescences or flowers were then bagged using wax paper bags.

In order to minimize damage to the open flowers in the cross pollination and self pollination experiments, two needles were used to remove the anthers and to dust the pollen grains on to the stigma. All the needles were cleaned with methylated spirit after each treatment to ensure that the needles were free from pollen grains before using them again. The treatments were considered successful when mature fruits could be collected from the inflorescences easily with a pair of forceps or from the wax paper bag. The mature fruits collected were then germinated in subsequent seed viability studies. The fruit set was determined by dividing the total number of fruits with the total number of flowers. For the statistical analysis, the percentage of fruit set among the treatments was compared.

C. Pollinating agents

The tagged flowers were observed for 15 minutes per hour from morning (approximately 0800 hour) to evening (approximately 1700 hour) at the study site. Insects that made contact with the flowers and their behavior were recorded and photographed. Flowers that had reached flower anthesis were stained in 0.01% neutral red as well as 1% Sudan III to detect the presence of osmophores and nectaries respectively. Osmophores were recorded as present if the flower stained deep red, and nectaries present were stained reddish orange. To locate nectar glands, freshly anthesized flowers were collected and dipped into the neutral red stain prepared by dissolving neutral red in distilled water (1: 10,000). The flower buds were submerged in the stain for two to three hours. Nectar glands were recorded as present when red spots were observed at the base of the filaments.

3.3.7 Seed germination.

The seeds of *A. sessilis* and *A. brasiliana* were small and they would probably be damaged if they were taken out from the fruit. Therefore, the whole fruit was used for germination instead of just the seeds. The germination site is located outside the green house of Institute of Biological Sciences. The mature fruits collected from the control experiments and pollination experiments were then sown on the soil surface in sowing plots of organic soil. The seeds were considered viable if germination was successful.

For labeling purposes in the germination experiments, aluminum tags were used. Observations were carried out daily to determine the incubation period and the time taken for the first appearance of the radicle on the soil surface were recorded. When a seedling had fully developed, the height of the seedling and the length of its root were recorded.

To calculate the percentage of seed germination, the total number of seeds sown was divided with the total number of germinated seeds. For the statistical analysis, the height of seedling was also compared.