CHAPTER 3

MATERIALS AND METHODS

Physiological studies of diploid and tetraploid *Hippeastrum* concerning aspects of varietal improvement were divided into 2 parts, 1) characteristical studies and 2) floral development investigations. The first part included the studies involving some morphological and anatomical characterizations for varietal identifications while the second part dealt with growth and development of floral buds leading to organogenesis patterns of floral parts and post-fertilization development of diploid/ polyploid embryos.

Hippeastrum used in these studies were those of 1) local and introduced diploids and 2) commercial tetraploids. Experimental varieties varied in accordance with specific objectives of each experiment. The research was conducted at Department of Plant Science and Natural Resources, Faculty of Agriculture, Chiang Mai University and Huai Hong Krai Royal Development Study Centre, Doi Saket, Chiang Mai, or stated otherwise for particular investigation. The experiments were carried out during the period of October 2008 to January 2011.

Part 1 Characteristical Studies

This part composed of three studies of *Hippeastrum* characterization, i.e. morphological, anatomical and cytogenetical. Materials and methods used in each experiment were as follows:-

Experiment 1: Pollen morphology

Morphological characterization of pollens were studied to express varietal identities of tested varieties. Techniques of pollen investigation under light microscope (LM) and scanning electron microscope (SEM) applied in this experiment were those suggested by Ruksat (1991) and Wongsawad *et al.* (1996).

1.1 Materials

1.1.1 Plant materials

Varieties of *Hippeastrum* used in this experiment were grouped as 1) **local diploids**: SP-Pink and SP-Red, 2) **introduced diploids**: Night Star and Tango and 3) **commercial tetraploids**: Apple Blossom, Lemon Lime and Susan. The plants of those varieties were obtained from the plant collection unit of Huai Hong Krai Royal Development Study Centre. Flowers of each variety were illustrated in Figure 1.

1.1.2 Chemical reagent is silica gel

1.1.3 Scientific apparatus

1.1.3.1 vials

1.1.3.2 gelatinous capsules

1.1.3.3 microscopic slides and cover slips

1.1.3.4 compound microscope (Olympus CX 31) with microscopic camera set

1.1.3.5 scanning electron microscope (SEM): JEOL Model JSM 5410 LV

1.1.3.6 gold coater: JEOL Model JFC-1200 Fine Coater with stub and carbon tape

1.1.3.7 miscellaneous: forceps, needles, paintbrushes and labels

1.2 Methods

Investigations of pollen morphology of the seven varieties were carried out aiming at observing and recording morphological microscopic characters of the pollen samples.

Pollens were collected from dehisced anthers of experimental plants then kept in gelatinous capsules. The capsules were consequently placed in bottles containing silica gels to keep away humidity.

In order to observe pollen morphology, extracted pollens were dispersed on microscopic slides already smeared with distilled water. Cover slips, were then placed over the pollens. Investigations of those pollens were done under light microscope. Shape and size of at least 20 pollens per sample were recorded. Microphotography was consecutively conducted.

SP-Pink

SP-Red

Night Star

Tango

Apple Blossom

Lemon Lime

Susan

Figure 1 Flowers of different varieties

Pollen surface was studied in details under scanning electron microscope following the techniques described by Ruksat (1991) and Wongsawad *et al*. (1996). Pollen samples were collected in the same manner as stated previously. They were then sprinkled evenly over the surface of the carbon tape secured on the specimen

stubs. The pollens were then coated with gold by JEOL Model: JFC-1200 Fine Coater. The stubs containing gold-coated pollens were inserted on the specimen stage of the microscope. Pollens of each sample were examined and recorded for their size and shape. Microphotographs were taken. Sculpture and structure of the exine as well as the aperture numbers appeared on the exine were then recorded and analysed, accordingly. Specific characteristics of the pollen representing different varieties were hence concluded. These investigations were done at the Electron Microscopy Research and Service Centre, Biotechnology Centre and Institute of Product Quality and Standardization, Maejo University, Chiang Mai.

Experiment 2: Anatomical characterization

Anatomical studies comprised tissue system investigations of various plant parts aiming at referencing particular characters of those tissue systems to indicate specific identities of experimented varieties. Meanwhile, indication of some physiological behaviors relevant to such characteristics of the tissues in different systems was also evaluated. Additionally, structure of the cells of some tissue systems, e.g. dermal tissue and vascular tissue could reflect capacities of those tissues concerning their display and post-harvest qualities. Materials and methods of this study were as follows:-

2.1 Dermal tissue

Particular epidermal cells function differently from the common ones. Their structures and occurance as well as density of those cells were supposed to reveal the plant identities at varietal level. This investigation was objected to observe characteristics of regular epidermal cells as well as stomata of different *Hippeastrum* varieties from the tissue sections mounted on permanent slides. The sections were those of leaf, perianth, peduncle and pedicel prepared through paraffin embedding methods proposed by Johansen (1940) and Sass (1966). Peeling off of epidermal layers of those organs was done following the methods stated by Chaidee *et al*. (1998), Sakultarl (2007) and Kermanee (2008).

2.1.1 Materials

2.1.1.1 Plant materials

Four varieties of *Hippeastrum* were studied in this experiment, namely SP-Pink and SP-Red, belonging to the diploid group and Apple Blossom and Susan of the tetraploid group (Figure 1).

2.1.1.2 Chemical reagents for epidermal layer extraction and mounting

2.1.1.2.1 acetic acid (CH_3COOH)

2.1.1.2.2 30% hydrogen peroxide (H_2O_2)

2.1.1.2.3 safranin O ($C_{20}H_{19}N_4Cl$)

2.1.1.2.4 50% glycerol ($C_3H_8O_3$)

2.1.1.2.5 clear nail polish

2.1.1.3 Chemical reagents for permanent slide preparation

2.1.1.3.1 formalin-acetic acid-alcohol (FAA) for killing and fixing

2.1.1.3.2 95% ethyl alcohol, absolute ethyl alcohol, tertiary butyl alcohol (TBA) and distilled water for dehydrating (Sass, 1966)

2.1.1.3.3 liquid paraffin

2.1.1.3.4 paraffin (Paraplast Plus®)

2.1.1.3.5 albumin adhesive

2.1.1.3.6 xylene

2.1.1.3.7 Delafield's hematoxylin, safranin and fast green

2.1.1.3.8 Canada balsam (Merck)

2.1.1.4 Scientific apparatus

2.1.1.4.1 compound microscope with microscopic camera set

2.1.1.4.2 stage micrometer

2.1.1.4.3 microscopic slides and cover slips

2.1.1.4.4 test tubes

2.1.1.4.5 glass bottles and vials

2.1.1.4.6 heat block and thermometer

2.1.1.4.7 desiccator

2.1.1.4.8 paraffin embedding oven (56 °C)

2.1.1.4.9 card paper for paraffin blocks

2.1.1.4.10 wooden block (1.5x1.5x1.5 cm^3) saturated with paraffin

2.1.1.4.11 rotary microtome of Leitz Wetzlar with microtome knife

2.1.1.4.12 microtome knife sharpener with fine and coarse abrasive

2.1.1.4.13 hot plate

2.1.1.5.14 staining jars

2.1.1.5.15 miscellaneous: alcohol flame, boxes of slides, droppers, forceps, labels, needles, paintbrushes, pencils, scalpel handles with blades

2.1.2 Methods

Epidermal cells were investigated superficially by removing the epidermal layers and inspecting the single layered tissue through light microscope using the methods stated by Sakultarl (2007) and Kermanee (2008). Permanent slides of transverse sections of these organs were also produced for microscopic investigation following the techniques of paraffin embedding (Johansen, 1940 and Sass, 1966). The organs being sampled for sectioning were leaf, perianth, peduncle and pedicel and the areas of sampling were indicated as seen in Figure 2.

2.1.2.1 Epidermis removal

As for surface layer peeling, 1 cm^3 pieces of the tissue were taken from the plant parts and macerated in a solution of 1:1 acetic acid and 30% hydrogen peroxide for 2-6 hours at 60 ºC, depending upon the texture of the specimens. Strips of upper and lower epidermis were removed with forcep and placed in a plate containing distilled water. They were then rinsed for 2-3 times, stained with safranin for 5 minutes and mounted in 50% glycerol. When possible, the samples were directly observed without any treatment.

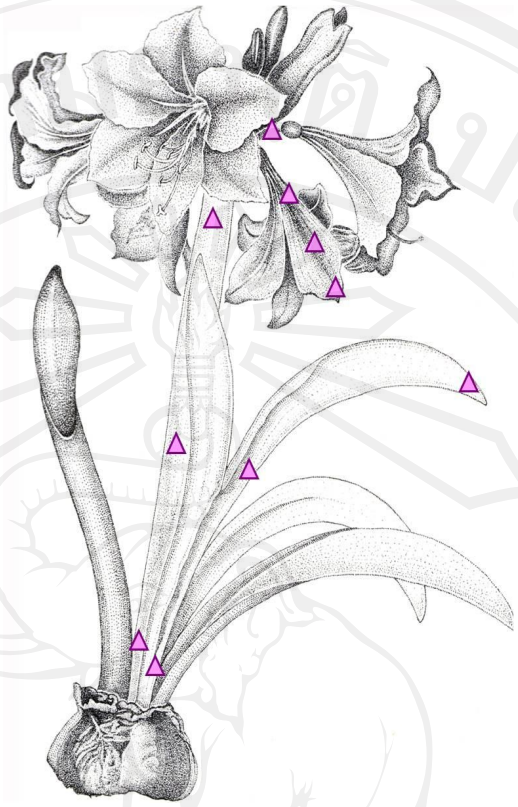

Figure 2 Drawing of a *Hippeastrum* plant marking the areas of sampling (△) for epidermal cell investigation

For stomatal observation, determination of 25 randomly selected stomata occurred on both upper and lower surfaces of each sample were inspected and the length of stomatal guard cells were microscopically measured via ocular micrometer. Similarly, 25 microscopic fields from each sample were randomly selected for evaluations of stomatal frequency and orientation. Recording of characteristics of subsidiary cells and neighbouring cells were also made (appendix A and B).

2.1.2.2 Transverse sections

Transverse sections of leaf, perianth and peduncle were prepared via paraffin embedding methods described by Johansen (1940) and Sass (1966) (appendix C). Permanent slides of the tissues were inspected under light microscope to record the size and shape of epidermal cells, including stomata.

2.2 Vascular tissue

Anatomical studies of vascular tissues of inflorescence stalks and pedicels could reveal the structures of vascular elements, especially those of supportive functions hence reflecting stalk toughness and transporting vessels relating water uptake of the stems. The sections were prepared via paraffin embedding methods as earlier mentioned in 2.1.2.2.

Experiment 3: Chromosome investigation

Generally, cytogenetical characterization of the plant is mainly evaluated through their karyotypic expression. An aspect of such expression is via chromosome number. In this experiment, investigation of root tip chromosomes of different varieties of *Hippeastrum* was thus conducted. Feulgen's squash technique (Chen, 1992) was modified by varying tissue sampling treatment, pre-treatment, maceration and staining to obtain the most suitable method being a protocol, applicable for various varieties of the plant.

3.1 Materials

3.1.1 Plant materials

Hippeastrum varieties used in this experiment were SP-Pink, SP-Red, Apple Blossom, Susan and the hybrids.

3.1.2 Chemical reagents

3.1.2.1 para-dichlorobenzene (PDB)

3.1.2.2 glacial acetic acid

3.1.2.3 95% ethyl alcohol

3.1.2.4 hydrochloric acid (1 N HCl)

3.1.2.5 carbol fuchsin

3.1.3 Scientific apparatus

3.1.3.1 vials and glass bottles

3.1.3.2 heat block and thermometer

3.1.3.3 microscopic slides and cover slips

3.1.3.4 compound microscope with microscopic camera set

3.1.3.5 needles and forceps

3.2 Methods

Bulbs of experimental varieties were planted in growing media to obtain the roots. Root tips of each variety were harvested when they were 10-20 mm long. The root samples were then run through the process of Feulgen's root-tip sqush for chromosome investigation modified by Chen (1992), Vitayasak (1996) Arayakitcha-roenchai (2000), Sudsa-nguan (2001) and Chawalid (2003).

The samples were taken at 7.30, 8.30, 9.30, 10.30 and 11.30 a.m. then pre-treated in aqueous solution of para-dichlorobenzene (PDB) for 24, 36 48 and 60 hours at 10 ºC. They were fixed in the solution of 1:3 acetic acid and alcohol for 5 minutes before being transferred into 70 % alcohol at 4 ºC or hydrolyzed in 1 N HCl for 5 minutes at 60 ºC. Prior to staining in carbol fuchsin for 6, 12 and 24 hours, the samples were rinsed in distilled water for 2-3 times. The roots were squashed on microscopic glass slides in a drop of carbol fuchsin then mounted with cover slips.

Inspection of chromosomes were done under light microscope. At least 25 cells at metaphase stage were examined for chromosome counts. Records and photographs were taken, accordingly.

Part 2 Floral Development

Evaluation of reproductive capacity of the plants can be compassed by examining the process of their floral development especially those of stamen and pistil, the organs involved in fertilization of the plants. This part of studies was hence carried out to observe such development of *Hippeastrum* varieties of different ploidy levels. The studies were allocated into 3 parts, 1) flower bud development 2) development of pollen and ovule and 3) embryo development.

Since the main objectives of this research were meant to support varietal improvement of *Hippeastrum,* especially the efforts of obtaining polyploid hybrids, therefore the trials in this experiment were extended to some physiological aspects concerning post-fertilization processes. Such trials involved those related to seed germination. Materials and methods of the experiment were as follows:-

Experiment 4: Floral bud development

Flower buds of various sizes were detached from the bulbs and fixed in FAA solution for anatomical studies. The buds were then passed through paraffin embedding process as done in experiment 2, hence producing permanent slides of transverse and longitudinal sections of those floral parts.

Studies carried out in this experiment fell into 2 categories. Firstly, investigation of floral development of the flower buds of different sizes and secondly, observation of developmental stages of flower buds situated at different scale axils of the same bulb. The latter was done prior to the planting season using the bulbs of different sizes in individual variety, grading as 10.1-12.0, 12.1-14.0, 14.1-16.0, 16.1-18.0, 18.1-20.0 and 20.1-25.0 cm in circumference according to The Flowerbulb Centre, Hillegom, Holland, British Standard Institution and American Association of Nurserymen (Arthey, 1975; Canadian Nursery Landscape Association, no date; Rees, 1972).

Experiment 5: Microsporogenesis and megasporogenesis

The aim of this experiment was to follow histological changes occurring during male and female organogenesis in the flower buds at different stages of development. Varieties of *Hippeastrum* and materials and methods for histological work used in this experiment were the same as in Experiment 4. Transverse and longitudinal sections of anthers and ovaries at different ages were inspected under microscope. Development patterns of such organs indicating fertilization of reproductive tissues were concluded, accordingly.

Experiment 6: Pollen viability and storage

6.1 Pollen viability

Pollen germination capacity is a usual way of analysing pollen viability. Pollen of experimental varieties were therefore tested in this experiment. Materials and methods of germination tests were as follows:-

6.1.1 Materials

6.1.1.1 Plant materials

Blooming flowers of varieties as stated in 1.1.1

6.1.1.2 Chemical reagents

6.1.1.2.1 1 % aceto-carmine

6.1.1.2.2 gelatinous capsules containing silica gel

6.1.1.2.3 pollen germinating solutions (Brewbaker and Beyong, 1963)

<u>stock mineral solution</u>

H_3BO_3	0.10	g
$Ca(NO_3)_2.4H_2O$	0.30	g
$MgSO_4.7H_2O$	0.20	g
KNO_3	0.10	g
H_2O	100	ml

<u>culture solution</u>

stock mineral solution	1.0	ml
sucrose	0.2-1.0	g
H_2O	9.0	ml

6.1.1.3 Scientific apparatus

6.1.1.3.1 glass bottles (vials) containing silica gel

6.1.1.3.2 incubator (5°C)

6.1.1.3.3 microscopic slides and cover slips

6.1.1.3.4 compound microscope with microscopic camera set

6.1.1.3.5 miscellanous: petri dishes, filter papers, droppers, glass rods, forceps and needles

6.1.2 Methods

Viability tests of the pollens were done using the methods described by Mahmoud *et al.* (1998), Choldumrongkul *et al.* (1996) and Arayakitcharoenchai (2000). Pollens from different treatments were collected from dehisced anthers at different times of the day, i.e. 6.00, 7.00, 8.00, 9.00, 10.00, 11.00 a.m., 12.00, 1.00, 2.00, 3.00, 4.00, 5.00 and 6.00 p.m. They were then cultured in liquid medium containing 3% sugar. Viabilities of the cultured pollen were tested in aceto-carmine

solution or in culture medium itself. Microscopic slides containing cultured pollens were placed on triangular bar of glass slides in a moist box at room temperature. Germination was inspected and recorded under light microscope. Data were collected on the number of germinating pollens vs non-germinated ones, appearing in the vicinity of 1x1 mm^2. Ten replicates of each treatment were taken. Germination percentages were then calculated. The pollen having its tube twice as long of its diameter was considered a viable pollen (Pearson and Harney, 1984). Absolute pollen viability, or the effective germination capacity was calculated using the formula suggested by Visser *et al.* (1977a, b)

Absolute pollen viability = % stained pollen x germinated pollen/100

6.2 Pollen storage

Trials of low temperature storage of pollens at 5°C, were done, comparing with those at the room temperature (28-33°C). The pollens collected from the plants were placed in gelatinous capsules with silica gel intact. They were taken out for viability tests periodically, i.e. at 0, 1, 3, 6, 10, 15, 21, 28, 36, 45, 55, 66, 78, 91, 105 and 120 days of storage. Such tests were done using the same methods as stated in 6.1.2. Determination of pollen viability under light microscope was judged from germinating rates of the pollens from different treatments recorded from 10 randomly selected microscopic fields.

Experiment 7: Embryo development

This experiment was objected to study development of the hybrid seeds obtaining from diploid and tetraploid crosses. Germinability tests of those seeds as well as anatomical changes of the intact embryos throughout germination process were conducted.

7.1 Germination capacity

7.1.1 Materials

7.1.1.1 Hybrid seeds from crosses of diploid and tetraploid parents as stated in Experiment 2.

7.1.1.2 sowing media of soil, sand, rice-husk charcoal and ground-nut husk (0.5:1:1:1 in volume)

7.1.1.3 plastic boxes

7.1.2 Methods

Hybrid seeds were germinated in sowing media in 2 manners, i.e. 1) direct seeding by sowing the seeds vertically or horizontally then covered thinly with the media and, 2) floating the seeds in water to sprout. Germinating percentage of the 2 treatments were recorded and germination behavior of the seeds were observed.

7.2 Development of hybrid embryos

This study concerned investigation of development of hybrid embryos through histological work. The embryos at different stages of development were removed from the seeds and fixed in FAA solution. They were consequently run through the process of paraffin embedding to produce permanent slides of transverse and longitudinal sections to be observed under light microscope. Materials and methods of permanent slides preparation were described earlier in Experiment 2.