CHAPTER 2

LITERATURE REVIEW

Hippeastrum is a monocotyledonous flowerbulb belonging to the family Amaryllidaceae. The name *Hippeastrum* derived from two Greek words, **hippeus**, meaning knight and **astron**, meaning star. It is an important genus of Amaryllidaceae, comprising 70 species, approximately (Rees, 1985). Read (2004) reported over 300 cultivars existed and most of the horticulturally significant were bred by Ludwig, Warmenhove and Van Meeuven of the Netherlands with a certain amount released by Hadeco Nursery (Barnhoorn) in South Africa.

The genus *Hippeastrum* can be considered a tropical plant by origin, as it is indigenous to Central and South America, centered in Brazil and Peru, distributing from Mexico to Chile and Argentina (Okubo, 1993). It is commercially known as Amaryllis while the true Amaryllis, i.e. *Amaryllis belladonna*, originating in South Africa, is not widely known in Thailand. These two plants can be distinguished from one another by two characters, i.e. the hollowness of scape and the existance of scales on the filament. Additionally, *A. belladonna* produces solid scape and scaleless filaments (Goldblatt, 1984; Traub, 1958).

Some *Hippeastrum* produce large and showy flowers with bright colours of, for instance, red, pink, orange and white while others are bi-coloured. The flowers are of several types such as cultivated wild, belladonna type, reginae type, leopoldii type, trumpet-flowered, orchid-flowered, miniature-flowered and doubled-type. They are usually grown as potted plants or as cut flowers. In subtropical and tropical areas the plants are as well used in landscaping (Okubo, 1993; Rees, 1992; Schulz, 1954; Vijverberg, 1981).

1. Botanical aspect of *Hippeastrum*

General description of *Hippeastrum* stated below are based on Akavipat and Maneerat (1979), Bailey (1949), Huxley (1992), Jintapakorn (1999), Keng (1969),

Ockenga (2002), Panusan (1993) and Singh (1972), or indicates otherwise.

1.1 Bulb

The bulb is tunicate in structure, comprising layers of swollen and succulent concentric scales with their bases attached to the basal plate, modified from underground stem, of thickend, cone-shaped with very short internodes. The scales themselves modify from leaf bases and store food materials. The bulb is round in shape. The outermost scale, called tunic, is dry, providing protection from drying out and mechanical injuries to the whole bulb. The apical bud of the basal plate is a leaf bud. Lateral buds appeared alternately on the basal plate, with the one next to the apical bud being a flower bud. Other flower buds occur at every forth scale axil away from the uppermost one. The lateral buds of the outer scales can develop into bulblets. *Hippeastrum* bulbs vary in size, depending upon age and type.

1.2 Root

Hippeastrum produces adventitious roots surrounding the lowest part of the basal plate. The root is round and slightly tapered towards the tip. Young roots are white and turn brownish when older. The tip of the roots usually branch out. The roots have dual functions of absorbing moisture and nutrients from the soil and of securing the bulb from tipping over as the inflorescence grows.

1.3 Leaf

The leaves are simple in type, linear- or strap-shaped. They are sessile or slightly petiolated and appear alternately. The leaf bases are sheathed and fold inwards while the upper parts expand towards the tip. The leaf obtains entired margin, acute apex and parallel veins with a prominent mid rib. The leaves, moderately succulent, are green in colour, but some varieties have cream or dark red area along the edges or at the tips, especially those having crimson flowers.

1.4 Flower

Hippeastrum produces umbel inflorescence. Each inflorescence holds 2-15 florets, depending on species and varieties. Inflorescence stalk or peduncle or scape is rather large and succulent. Some are round and hollow. The scape, covering with fine powdery bloom, can be light or dark green in colour. Bloomed and bracteolated pedicels of round or slightly angular are equal in size. The flower buds are

wrapped inside 2 spathe valves. These valves, greenish-white, yellowish or pinkish while closed, light brown and papery when old, split open as the bud increases in size.

Florets are bisexual, each bearing a receptacle. Sepals and petals of each floret look alike, thus, called perianths. They are 6 in number with their bases fused together forming a perianth tube. The upper parts of this perianth tube separate and spread out in a shape of funnel, so called the perianth segs. The perianths, collectively called perianth, compose of 2 whorls, each of 3 in number. The outermost perianths, arranged alternately with the innermost ones, are slightly wider. The shape of the perianths is elliptic, broad at the middle and narrow both at the tip and the base. Each perianth obtains a ridge or keel at the centre. The colour of those perianths are of wide range, from pastel pink to vivid orange and from clear white to velvety red, some are glossy and glow in sunlight. Flowers of some species can be fragrant. Although perianths are applied as botanical terms for collective perianths of *Hippeastrum* but the word petal is as well used otherwise.

Hippeastrum floret contains 6 stamens and a pistil. Each stamen is made up of a filament stalk and a two-lobed anther. Each anther lobe bears 2 pollen sacs holding yellowish pollen grains within. The pistil has a trifurcate capitulum stigma on a long style with inferior ovary at the basal end. The ovary contains 3 locules. Each locule embodies several ovules. The ovule placentaion is that of axile.

The fruit of *Hippeastrum* is a type of loculicidal capsule, having 3 locules. The seeds are rather large, flat and papery, with the shape of the italic letter *D*. Each seed contains succulent endosperm. The colour of the seed is usually black or dark brown. They have no dormant period. The germination type is epigeal.

2. Growth of *Hippeastrum*

Le Nard and De Hertogh (1993) mentioned that in natural habitats, geophytes are subjected to a wide range of climatic conditions, thus, they are characterized by marked seasonal changes in temperature, rainfall, daily radiation and photoperiod. It was also stated by them that an exception to such situation would be the equatorial and subtropical areas where more or less uniform conditions are present all year round at a given altitude, thus many genera do not show any eminent rest period and continuously posses foliage leaves. Accordingly, *Hippeastrum* was then considered

by Hartsema (1961) and Rees (1972) being one of those genera, along with *Clivia*, as a non-deciduous bulb. But, it was also stated on the contrary that once *Hippeastrum* are planted under different conditions from its native habitat the rest period can then be observed. Such rest period can also be resulted from the application of techniques benefiting commercial production of the plants.

Since climatic conditions as well as seasonal changes play important roles towards the annual growth cycle of *Hippeastrum* and other bulbous plant genera (Rees, 1972), it is, hence, worthy to review further on those aspects, particularly those of bulb dormancy/rest period. Such reviews would provide better understanding of *Hippeastrum*'s nature of growth since these certain growth phases have been at times discussed, as a part of the growth phase of *Hippeastrum* under certain circumstances.

Concerning physiology of bulbous plants, it was stated by Le Nard and De Hertogh (1993) that in climatic areas with marked seasonal changes, bulbs developed mechanisms to survive adverse climatic conditions constituted by low or high temperatures and/or drought. Under such conditions, bulbs developed a state of rest, in which they did not show any visible external growth.

Expression of the rest period were reported to be different among the flowerbulbs of different species. The bulbs that showed their active growth and flowering in spring generally exhibited their rest period in summer when the temperatures were high and the soil was dry. The plants resumed their growth in autumn. These flowerbulbs which required a warm-cold-warm sequence to express active growth and complete their annual growth cycle included *Hyacinthus*, *Narcissus* and *Tulipa*. The summer flowering bulbs, having an active growth during summer and a rest period in winter when the temperatures were low, need a low-warm-low temperature sequence to express their active growth and complete their annual growth cycle. The genera included were *Allium* and *Lilium*, for instance. A genus *Haemanthus* also belonged to this latter group although consi- dered an evergreen plant because sometimes it behaved as summer- or winter-growing species (Le Nard and De Hertogh, 1993).

It has been referred that during the rest period various bulb genera show a great diversity of behavior. As for *Crocus*, *Hyacinthus* and *Tulipa* their active organogenesis, i.e. flower bud differentiation, vegetative bud differentiation and root

initiation, take place during the rest period while in other genera, e.g. *Lilium*, organogenesis is reduced or even temporarily arrested. Such diversity of growth and development including the existence of specific organogenesis processes occurred during the rest period have resulted in wide debates regarding the subject of bulb dormancy. Thus, different concepts over the matter have been proposed by various scientists, basing their approaches on the definitions of dormancy (Le Nard and De Hertogh, 1993).

Amen (1968), cited by Le Nard and De Hertogh (1993), considered the ability of the shoot to develop new organs and to perform elongation as the main criteria, divided major bulb species into three groups. The first group which exhibited dormancy included *Allium*, *Gladiolus* and *Lilium*, having relatively long period during which differntiation of new organs and their elongation were totally interrupted. The second group fell onto *Hyacinthus*, *Narcissus* and *Tulipa*. Dormancy of these bulbs was very slight since flower bud differentiation took place immediately after bulb formation. Dormancy was restricted to the flower stem which could not rapidly elongate in the absence of an extended cold treatment. Finally, the third group of Dutch Iris showed no dormancy unless it was imposed.

Rees (1981) mentioned his topic of bulb dormancy, based on *Tulipa*, regarding the periods of meristem inactivity as true dormancy while Le Nard (1983), working on Dutch Iris, *Gladiolus* and *Tulipa*, suggested that the term dormancy could be applied to the entire bulb not specifically the apical bud or the meristem. The period of bulb dormancy should be the period during which the bulb was not able to react to environmental factors and organogenesis was not active.

Referring to the works stated above, Le Nard and De Hertogh (1993) concluded the definition of dormancy for bulbs as a complex and dynamic physiological, morphological and chemical states during which there was no apparent external morphological changes or growth. Internally, many physiological and/or morphological events were occurring. This state could also refer to as “Interbulb Development Period” indicating that as soon as some external changes were observed, e.g. root growth on the basal plate or obvious shoot growth, the bulbs were no longer dormant. Thus, some growth processes could be rapidly observed provided the bulbs were placed under favourable environmental conditions.

Since the bulbs are reported as never physiologically or biochemically at rest, it is important to realize the precise effects of environmental factors on physiologically processes to put into practices the techniques that can effectively control bulb growth (Le Nard and De Hertogh, 1993) of which have been the subjects of research being carried out by several institutes.

As for *Hippeastrum*, because of its tropical origin, it is assumed to have no real dormant period in the annual cycle of growth and development. It is then considered having no growth periodicity under optimal climatic conditions. But, wide climatic changes and horticultural practices can apparently impose the periodicity on the plant (Le Nard and De Hertogh, 1993).

Hippeastrum in tropical rain forests are observed to grow year-round with alternating vegetative and reproductive stages. Since *Hippeastrum* is of tropical origin, the ancestor plant should not have needed to evolve the phenomenon of bulb formation if the environments had been fairly constant without considerable fluctuation of temperature, day length, rainfall, etc. Thus, some unfavourable factors must have compelled those ancestor plants to form the bulbs for survival (Okubo, 1993).

Nevertheless, although as it has been presumed that *Hippeastrum* has no real dormancy, Okubo (1993) literally proposed that either the induction of bulb formation and that of dormancy were the same phenomenon or that the induction of bulb formation was the first process of dormancy induction. In such concept, the author suggested that the induction of bulb formation could be referred to the pre-dormancy stage. Thus, it could also be considered as the only difference in the degree or intensity of the true dormancy stage of the plant.

On practical sides, Okubo (1993) and Zhang and Pinfang (1995) reported the growth of *Hippeastrum* under suitable conditions of fairly high temperature and adequate humidity that within one year the plant concieved three cycles of growth and flowered three times. Doorduin (1990) studying the growth and development of two *Hippeastrum* varieties, namely Apple Blossom and Red Lion, under greenhouse conditions found that the plants could grow all year-round and could be forced to bloom off season.

De Hertogh and Gallitano (2000) working on the variety Apple Blossom could produce the plants with the highest quality under 22/18°C (day/night) with long days.

These plants reached the market stage in 25 days and achieved the full blooms in 32 days. The lowest quality plants were obtained at 30/26°C under both short and long day photoperiods of greenhouse conditions. Plants grown at 30/26°C were very short and produced small pale flowers, indicating inadequate pigmentation of the petals.

3. Breeding of *Hippeastrum*

Meerow stated in 2009 of a significant contribution of the Netherlands and South Africa, over a 200-year breeding history, in releasing popular large-flowered hybrids of *Hippeastrum*, currently dominating the world market. Huxley (1992) and Ockenga (2002) indicated several peaks of enthusiasm of *Hippeastrum* hybridization during the eighteenth to twentieth centuries, with the first hybrid being *Hippeastrum* x *johnsonii* from the cross of *H. reginae* of Southern America's and *H. vittatum* of Central America's, in 1799 by A. Johnson. Okubo (1993) mentioned more about interspecific hybridization of *Hippeastrum* among *H. aulicum*, *H. correiense*, *H. elegans*, *H. reticulatum*, *H. rutilum* and *H. stylosum*, especially the crosses using Peru's species of *H. leopoldii*, *H. pardinum* to produce large-flowered cultivars bred in England since 1865 which were first released around 1870.

The long history of *Hippeastrum* hybridization in the Netherlands began since then, using Brazilian species of *H. aulicum*, *H. retinae*, *H. reticulatum*, *H. rutilum* and *H. vittatum*, and Peruvian's of *H. psittacium*, *H. leopoldii* and *H. pandinum*. Khaleel and Siemsen (1989) and Rees (1992) reported some 250 cultivars available commercially. New hybrids have also been developed in South Africa, North America, Australia, Japan, India, Israel, and Brazil, according to Ockenga (2002).

It can be seen from above that interspecific hybridization has been carried out in many breeding institutions to enlarge genetic diversity of commercial materials using wild species, mostly those of diploid with a few natural tetraploid (Meerow *et al.*, 1992). Achievements have been made based on specific purposes of individual scientist or institution, choosing particular species of genetic variates to serve their breeding programmes. Meerow (2009), for instance, focused heavily on *H. papilio* and *H. brasilianum*, of which provided him over 1,000 selection progenies of diploid, triploid, and tetraploid. Meanwhile, a research geneticist at the USDA's Subtropical Horticulture Research Station in Miami reported success of producing hybrids from

H. papilio with choices of appropriate characteristics of compact growth, evergreen foliage, and long-lasting flowers of unusual colour range (Ockenga, 2002).

Attempts to develop commercial varieties have also been made by private companies in the United States, the Netherlands and South Africa who have been distributed a fair share of valuable hybrids of a great diversity in flower types, colours and shapes as well as stem lengths (Benschop *et al.*, 2010).

While commercial production of *Hippeastrum* expands extensively, varietal improvement of the plants is naturally necessitated to cope with demands in horticultural fashion and also those from the growers for environmental tolerance and long lasting shelf life. Rees (1992), looking onto breeding of *Hippeastrum* judged by concerned institutions on several criteria, described the basis for modern cultivars from which initial selections were made to be improved mainly on the premise of appearance, e.g. flower size, shape and colour, stem length, foliage colour and marking, time of flowering, ability to induce flowering, quality in preservation of cut flowers, and, in particular, flower fragrance. More importantly, tolerance to environment, diseases and pests were also one of the prime priorities since the number of approved and effective fungicides was diminishing, as stated by Okubo (1993).

As hybridization of *Hippeastrum* is actively in progress, according to the information reviewed above, suggestions have been made by many scientists. Okubo (1993) stated interesting goals and specific problems of *Hippeastrum* hybridization and suggested more consideration on investigating wild species in their natural habitats to be used in cross breeding with the known species or cultivars to improve genetic variations, especially those of environment hardiness, fragrant flowers and specific flower colours. He also mentioned that some intergeneric between Amaryllidaceae species, e.g. *Hippeaskelia* (*Hippeastrum* x *Sprekelia*), *Amanerine* (*Amaryllis belladonna* x *Nerine bowdenii*) and *Amarcrinum* (*A. belladonna* x *Crinum moorei*) should be tested and hybridized with *Hippeastrum* spp. to bring about wider variations. Since incompatibility may occur among the crosses, embryo culture or other biotechnological techniques should be studied alongside to overcome such problem.

Consideration of intergeneric hybridization of *Hippeastrum* as well as the problems arising on the matter were consequently answered. Benschop *et al.* (2010), referring to Sandler-ziv *et al.* (2004), indicated that wild relatives of *Hippeastrum* were

hybridized with *Hippeastrum* in Israel and the techniques of *in vitro* embryo rescue succeeded in saving the hybrids.

Cytological work appears to complement breeding research of *Hippeastrum* both in interspecific and intergeneric hybridizations. Meerow *et al.* (1992) reported that ploidy levels of *Hippeastrum* most available in natural state were diploid (2n=22) while a few were natural tetraploid (2n=44). He also reported success in tetraploid crosses as being very high, while those among/with the diploids were faced with particular problem of self-sterility. Arayakitcharoenchai (2000), Chawalid (2003), Patanakanog (1999) and Sudsa-nguan (2001) supported his findings stressing on the similar problem of self-incompatibility occurring in specific parents.

4. Plant characterization

Thatsaneeyakorn (2000) pointed out the significance of morphological and anatomical studies revealing specific characteristics of certain plants. Information obtained from such studies could be worth recording in the databases, applicable for the plants identifications. In improvement and development of plant varieties, specific characteristics of varieties involving those of the parent plants and of their progenies are required for evaluation of their genetic relationship. Meaningfully, characteristical identification is useful when varietal registration of the hybrids is required (Jones and Luchsinger, 1979; Junsongduang, 2003; Stuessy, 1990).

Specific characterization includes, for instance, comparative anatomy of plant parts, embryo types (Jones and Luchsinger, 1979), pollen morphology (Jones and Luchsinger, 1979; Ruksat, 1996), number of stomata, length of stomata and guard cells and density of epidermal cells on the surface of specific plant organs (Krishnaswami and Andal, 1978). This characterization is even more substantial when implies to plants of different ploidy levels. Moreover, characterization by means of cytotaxonomy, chemotaxonomy, biochemical systematics, and comparative phyto-chemistry can provide prominent identity of the plant varieties other than taxonomical identification (Chengkun *et al*., 1995; Jones and Luchsinger, 1979).

Recently, Wongpiyasatid *et al.* (2005) indicated that some biological characters such as stomata size, stomata frequency, leaf index value, pollen grain diameter

and other morphological characters are conveniently used in primary classification of the plant progenies having different ploidy levels.

4.1 Pollen morphology

Erdtman (1972), Moore *et al.* (1991) and Simpson (2006) described palynology as a study of spores and pollen grains. The subject concerns structures and formation of those organelles, their dispersal and their preservation under certain environmental conditions (Moore *et al.*, 1991). Spores and pollen grains possess a number of morphological and ultrastructural features. These palynological features have provided abundant characters that have been important in inferring phylogenetic relationship among the plants. In addition, the outstanding feature of mature pollen grain is its wall, which is sculptured in a great variety of patterns (Esau, 1977). Thus, features of spores and pollen grains are often used to identify a particular plant taxon (Simpson, 2006).

Pollen morphology is the study of external features and internal structure of the pollen, pollen development from early stage to maturity, changes in growth and development of the plants, relation of plants to their ancestors and evolutionary relationships among groups of plants (Ruksat, 1991). This study provides understanding of the functions of pollen, such as pollination biology and interactive relations. In addition, pollen identification is considered an exclusive fundamental of palynology (Ruksat, 1991; Shivanna, 2003).

4.1.1 Applications of pollen morphology

Agashe and Caulton (2009) referred to pollen morphology as a tool used for reliable identification of plant species, supplementing those of taxonomial. It has been widely used by systematic botanists as the characters for plant identification at higher taxonomic levels, applicable for phylogenetical research (Kantachot, 2008). Pollen characteristics can specify varietal characters of plants and are often readily identified to the genus level, sometimes to the species, if adequate reference material is available (Cutler *et al.*, 2008). Similar to other plant parts, pollen characters are so varied that the classification system of plants can be built up entirely on the basis of pollen morphology. Some of these pollen morphological characters are catagorized as symmetry, size and shape, pollen wall, exine stratification, ornamentation, furrows/ grooves and apertures. The last three characters are said to be very useful in basic

identification and classification of pollen (Agashe and Caulton, 2009). Accordingly, Moore *et al.* (1991) agreed to the identifiable characters of pollen morphology of shape, texture, aperture and exine, being used in classification of plants. These characters were thus established in keys to pollen and spore. Moreover, pollen morphology was recognized by researchers of its great importance in taxonomy, applied botany, i.e. plant breeding, embryology, plant physiology, genetics, plant evolution, as well as in agriculture, foresty, geography, ecology and economics. Recognitions were also included in palaeobotany, phylogeny, aeropalynology, biotechnology, microbiology, pollen allergy and so on (Agashe and Caulton, 2009; Faegri and Iversen, 1989; Ruksat, 1991).

4.1.2 Techniques in pollen morphology

Agashe and Caulton (2009) and Faegri and Iversen (1989) indicated that morphological characters of pollen can be studied if proper techniques are employed. Pollen grains could be obtained from different sources, i.e. from living plants, from herbariums and from fossils. The classical method of studying morphology of pollen has been through acetolysis, of which is done in serial steps. First, the pollens are collected from the source and stored in vials. These pollens are consequently kept at low temperatures or stored in chemicals such as 95% ethyl alcohol, glacial acetic acid, 4% paraformaldehyde or Karnovsky's fixative, if kept at room temperature. Subsequently, they are subjected to the standard technique of Erdtman's acetolysis (Agashe and Caulton, 2009; Erdtman, 1972; Faegri and Iversen, 1989; Kermanee, 2008; Moore *et al.*, 1991; Ruksat, 1991; Shivanna, 2003). The protoplast, the intine and the pollen coat substances of the pollens are thus removed by the fixative, leaving only the exine. The acetolyzed pollens then show their surface features such as ornamentation, apertures and exine stratification (Agashe and Caulton, 2009; Shivanna, 2003). The reagents used in acetolysis, i.e. concentrated sulphuric acid and acid anhydride, are not only corrosive but also react vigorously with water, hence precautions are seriously taken during the practice, and thus, the pollen samples must be dehydrated before being subjected to acetolysis (Moore *et al.*, 1991).

Morphological criterias required in identification of pollen taxa are very subtle and often difficult to perceive under light microscope. Thorough staining helps increasing the contrast of exine features, facilitating microscopic work. Pollen exines absorb certain stains more strongly than other substances present in the

preparation, and are therefore easier to detect in a stained mount (Faegri and Iversen, 1989). The most widely used stain for this purpose is aqueous safranin, but alternatively, fuchsin is also acceptable (Moore *et al.*, 1991).

To obtain the best possible details, Agashe and Caulton (2009), Erdtman (1972), Faegri and Iversen (1989), Kermanee (2008), Moore *et al.* (1991), Ruksat (1991) and Shivanna (2003) suggested that the pollen grains should be mounted in an embedding medium of proper refractivity. The most commonly used mounting media have been glycerol, glycerine jelly and silicone oil. Microscopic slides could be prepared temporary or permanently, depending upon the mounting media being used. Information of pollen wall structure at different levels can be obtained from acetolyzed pollen through optical sectioning, but, sections of the pollen grains produce via microtoming provide better features of exine elements (Shivanna, 2003). Techniques for specimen preparation for such purposes were readily described by Agashe and Caulton (2009), Johansen (1940), Keating (1996) and Sass (1966). However, procedures of collecting and preparing those specimens have to be adjusted in accordance with the plant species and the objectives of the studies.

The precision with which pollen grains can be identified depends, in part, upon the quality of the microscope used for observation (Moore *et al.*, 1991). Although light microscope serves well for modest morphological investigations (Faegri and Iversen, 1989) but very fine details of the objects could only be obtained under powerful microscopes. Moore *et al.* (1991) stated that electron microscope utilizes a beam of electrons rather than light for illumination. The beam has a much shorter wavelength, greatly increasing the resolution of the microscope. In recent years, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) have provided powerful techniques for studying the finer details of the exine (Shivanna, 2003). The exine stratification is best studied by making ultra-thin sections and examining them under TEM while the surface of the exine is three-dimensionally viewed with precise details from SEM (Agashe and Caulton, 2009).

Various techniques proposed by reseachers are found to be most suitable for preparation of pollen grains for TEM and SEM studies. Some recent examples are reported to be plant species belonging to the families Amaryllidaceae, Iridaceae, Ixioliriaceae (Dönmez and Işik, 2008), Callitrichaceae (Cooper *et al.*, 2000),

Celastraceae (Perveen and Qaiser, 2008), Compositae (Bunwong and Chantaranothai, 2008), Polygalaceae (Krachai *et al.*, 2009) and Zingiberaceae (Sotthikul and Apavatjrut, 2003). Most technicians prepared specimens via acetolysis method, dehydrated and air-dried, then placed on the stub with adhesive tape. The pollen grains are to be coated with heavy metal, e.g. gold, palladium or gold-palladium mixture before being investigated under SEM. However, coated fresh pollens can directly be placed on the stub without acetolysis and examined under SEM (Celik *et al.*, 2005; Wongsawad *et al.*, 1996).

Palynological studies involving species of Amaryllidaceae are scarce (Alves-Araújo *et al.*, 2007). Erdtman (1972) illustrated the pollen morphology of several taxa, distributing among several families of angiosperms, including approximately 90 species in 60 genera of classified Amaryllidaceae. Among these are *Crinum* (Erdtman, 1972; Huang, 1972), *Galanthus* (Dönmez and Işik, 2008; Sahin, 2000), *Hippeastrum* (Alves-Araújo *et al.*, 2007), *Hymenocallis* (Huang, 1972; Meerow and Dehgan, 1985), *Leucojum* (Dönmez and Işik, 2008), *Lycoris* (Ren *et al.*, 1995), *Narcissus* (Chen and Ueda, 1977; Dönmez and Işik, 2008) *Pancratium, Sternbergia* (Dönmez and Işik, 2008) and *Zephyranthes* (Alves-Araújo *et al.*, 2007).

One of the most important pollen morphological studies that can be cited for monocots was developed by Zavada (1983). In his studies, pollen morphology of representative species from various orders were compared. Suggestions of using aperture patterns and other ornamentation of the exine were proposed to obtain relationship of the lines. Following this study a few investigations of pollen characteristics of Amaryllidaceae from South America were done, objected on classification of the plants. Meerow and Dehgan (1985) analysed the pollen grains of *Hymenocallis quitoensis* through exine structure and its taxonomic implications. Later in 1988, they studied the pollen of the species belonging to the Tribe Eucharideae occurring in the Amazon region. Meerow, in the following year, had a check on pollen morphology of *Caliphruria*, *Eucharis* and *Urceolina*, examining various morphological and anatomical characters of theirs.

4.2 Dermal tissue

Dermal tissue system of the plants comprises the epidermis which is the primary outer protective covering of the plant body or the periderm which is the

protective tissue that supplants the epidermis, mainly found in the plants that undergo a secondary increase in thickness (Evert, 2006). The epidermis consitutes the outermost layer of cells of the leaves, floral parts, fruits, seeds, stems and roots before they precieve considerable secondary thickening. Functionally and morphologically, the epidermal cells are not uniform and among them, apart from the ordinary cells, many types of hairs, stomatal guard cells and other specialised cells are found (Fahn, 1974).

Esau (1977) and Evert (2006) described stomata as the pores formed by a pair of specialised cells called guard cells, which by changes in shape, bring about the opening and closing of the pore. The stoma may be surrounded by cells that do not differ from other ground cells of the epidermis. Contrariwise, in many plants the stomata are flanked or surrounded by cells that differ in size, shape, arrangement, and, sometimes in content, from the ordinary epidermal cells. These distinct cells are called subsidiary cells of the stoma. They participate in the osmotic changes involved in movements of the guard cells. Hetherington and Woodward (2003) and Willmer and Fricker (1996) supplemented that stomata control two major processes in the plant, photosynthesis and transpiration. Scientific interests have been made on the unique characteristics of stomata since the first reports of stomatal observations in the 1600s.

Stomata are found in the surface of arial parts of most higher plants. They are present in floral parts, including petals, sepal, stamens and gynoecia, and also in awns, paleas and lemmas of cereal inflorescences. In other plant organs including rhizomes stomata also appear, such as in the inner and outer surfaces of pods and fruits including the skins of banana and the surface of seeds. More typically, they are found in the surfaces of leaves, green stems and modified leaves such as bracts and tendrils. But, stomata are not found in the chlorophyll-free plants. In albino plants, though stomata are also found in locations stated above, e.g. petals, stamina filaments, carpels and seeds, of which are non-photosynthetic functional (Fahn, 1974; Willmer, 1983).

4.2.1 Applications of stoma morphology

Structure, type, size and number of stoma can be used in identification and classification of plant species (Kermanee, 2008). Classification of stomata

serving taxonomic purposes is made in accordance with the arrangement of neighbouring cells around the guard cells (Willmer, 1983).

Dore and Essad (1996) claimed that specific varietal characters of plants could be classified from stomatal size and shape, number of chloroplasts in guard cells, length of guard cells, ratio between dimension of subsidiary cells, number of epidermal cells, stomatal index and orientation and density on epidermal layers of the plants.

There are very few reports indicating studies on stomata of monocots. Cheadle (1953) examined stomata in Agavaceae, Alismataceae, Amaryllidaceae, Araceae, Butomaceae and Cannaceae while Stebbins and Khush (1961) and Fahn (1974) did on some members of Cyperaceae and Gramineae. Abid *et al.* (2007) carried out an investigation of stomatal types in Commelinaceae, Cyperaceae, Gramineae, Hydrocharitaceae, Juncaceae, Liliaceae, Najadaceae, Potamogetonaceae and Typhaceae.

Przywara *et al.* (1988) mentioned that several stomatal features were often used as morphological markers to identify ploidy levels of many plant species such as kiwifruit, cotton (Krishnaswami and Andal, 1978; Wongpiyasatid *et al.*, 2005), coffee (Mishra, 1997), anthrurium (Junpugdee and Te-chato, 2008) and orchids (Chidburee *et al.*, 2008). Explicit understanding of the relationship among ploidy levels of those plants were also obtained from such studies. Nevertheless, Dore and Essad (1996) recalled that the above mentioned parameters are not applicable to all species.

Evans (1955) and Speckman *et al.* (1965) stated that the length of stoma was the prominent indicator of polyploid levels in many plants, but accuracy of this characteristical identification would be at most, provided it was done alongside with anatomical study of the plants (Sarawichit *et al.*, 2006). In addition, observations gathered from stoma morphology and stomatal appearances of the plants under different growth conditions could reveal their certain behaviors, applicable for physiological analyses (Kermanee, 2008).

4.2.2 Techniques in stoma morphology

Researchers have contrived stomatal techniques from time to time to obtain accurate data on stomatal characteristics and stomatal behavior in various

aspects. Several methods have been developed to study stomatal morphology. Common methods include epidermal peels, impression of the organ surface and maceration of epidermal tissue. Light microscopy (LM) and SEM are used accordingly (Hultine and Marshall, 2001). However, in some cases suggestions are made to practice paraffin embedding technique to prepare longitudinal sections of concerned tissue for stomatal viewing. Anyhow, each of these methods has unique strengths and weaknesses that must be taken into account, depending on the species and the experimental goals (Gitz and Baker, 2009).

4.2.2.1 Peeling off method

Aiamla-or (2005) practiced the peeling of epidermis from lamina surface using a fine scalpel. The peel was placed on a glass slide with a drop of water or glycerol on the surface and then mounted with a coverslip. She claimed this practice as a simple, convenient, rapid and easy procedure. Stomata extraction had been done in many plants such as *Curcuma*, some species of Dipterocarpaceae (Srinual *et al.*, 2009) *Gossypium* (Wongpiyasatid *et al.*, 2005) and *Coffea* (Mishra, 1997). However, epidermal layers of particular plants are difficult to peel, especially those of grasses. Therefore, special treatments such as scraping of epider- mal layers, leach and stain technique, etc., would need to be applied to make the extraction of stomata possible. Anyhow, all those procedures are not always successful since parts of the leaf segments are unavoidably destroyed (Hilu and Randall, 1984).

4.2.2.2 Impression method

Impressions of the epidermis surface is accomplished when the epidermis is brought into contact with a viscous gel or a putty which is allowed to harden. Methods using silicone rubber, agarose gel, thin plastic strips softened with solvent, dental putty or clear nail polish are included (Gitz and Baker, 2009; Hilu and Randall, 1984). The impression of stomata made by a film of clear nail polish directly applied on epidermis surface was found convenient in recently years. Abid *et al.* (2007) indicated that the impression left on the polish film produced an excellent detailed image of the epidermis. Prabhawit (1988) also found the same results with coffee while Permlap (1996) experienced it with aromatic plants of Amaryllidaceae. Chaidee *et al.* (1998) announced her finding with water hyacinth

and Abid *et al.* (2007) and Perveen *et al.* (2007) did with monocots and dicots within flora of Pakistan.

4.2.2.3 Maceration method

Hilu and Randall (1984) and Kermanee (2008) described maceration technique used for obtaining epidermal layers of plant parts for stomatal observation. The technique included imbibition of the plant segments in a solution of nitric acid, potassium chlorate, acetic acid and hydrogen peroxide, or else, followed by heating the solution to near boil. The tissue was later recovered and the epidermis was stained. This method, although useful, is lengthy and requires some adjustment of the solution concentration according to the delicacy of the plant parts to produce intact epidermis. In addition, most care must be taken since heating concentrated acids to a near boiling point on a flame is strongly considered hazardous. Nevertheless, Przywara *et al.* (1988), Charoenchai (1998), Lemos Filho and Isaias (2004), Adedeji *et al.,* (2007) and Sakultarl (2007) revealed successes in studying stomatal characteristics of several plants using the maceration technique.

4.3 Anatomical characterization

Plant anatomy is a subject in plant biology that deals with the structures and development of seed plants. As for the structures of the plants, this subject reveals that internal organization of the plant bodies consists of cells and tissues of which the latter themselves are composed of groups of cells. Plant anatomy is in fact about evolution of development of structure, complexity and remarkable orderliness in the organization of the plant (Esau, 1965). Cutler *et al.* (2008), working on plant systematics, stated that external morphology is adequate to identify plant species botanically, provided that the complete plant specimens are available. But, he as well added that when morphological features of the plants are indistinct anatomical characters can fit in to help identify them with certain accuracy, since anatomy of the plants, according to Esau (1965), is a result of evolutionary specialisation of a long duration.

4.3.1 Applications of plant anatomy

Plant anatomy, as again stressed by Cutler *et al.* (2008) and Kantachot (2008), provides very useful information for identification and classification of plants. Stuessy (1990) added that anatomical data are often useful in solving

problems concerning genetic relationship among the plants since it offers evidences on homologies of morphological character status, capable of providing evolutionary interpretation.

Studies on anatomical characteristics of the plants are commonly carried out in serveral plant parts, e.g. leaf, root, stem, flower, fruit or seed, through processes of comparative histology, as done by Junsongduang *et al.* (2002), Kantachot (2008), Kharazian (2007), Lakoet (2004), Talingtaisong (2008) and Thiti- metharoch and Thammathaworn (2007). Many workers studied anatomical charac- teristics of plants to support their findings concerning various aspects of growth and development of several plants (Arayakitcharoenchai, 2000; Buathong, 2008; Chankhum, 2001; Chawalid, 2003; Chidburee, 2008; Hannantavivat, 1998; Jintapakorn, 1999; Kijkar, 2001; Kumsiri, 2002; Mongkolrattanasit, 2001; Phetchaburee, 1994; Puangkaew, 2001; Pubuopiend, 1992; Samatthiya, 2000; Sudsa-nguan, 2001; Surajid, 2001; Suraporn-piboon, 1996; Vitayasak, 1996 and Vutthijumnonk, 1990) and orchids (Chanaken, 2007; Fupanya, 2008; Klongklaw, 2008; Phornsawatchai, 2008; Prarasi, 2006; Saisuwan, 2008; Stern and Judd, 1999; Sukkasem, 2007; Thahanthai, 2010; Thainurak, 2008; Thongsan, 2008; Uncharisanggard, 2008).

Nevertheless, it was said by Jones and Luchsinger (1979) that the use of anatomical features in taxonomic classification/identification of the plants are yet made complete with other characteristics of morphology, palynology, embryology and biochemistry.

4.3.2 Techniques in plant anatomy

Investigation of internal structures of the plant is generally done under microscopy. Very thin sections of plant parts are required to allow precise inspections. Most scientists prefer to prepare their plant materials via free-hand sectioning since it is simple, takes shorter time and inexpensive, provided that the scientists themselves are skillful enough to handle such practice and that the plant specimens are not too hard nor too tough (Berlyn and Miksche, 1976; Ruzin, 1999). This simple technique of sectioning has been so popular that several researchers have worked on special techniques to enhance its effectiveness as seen from Lux *et al.* (2005) who successfully developed the clearing procedure of the free-handed sections using lactic acid. He practically used the technique in combination with fluorescence

microscopy to study histological development and the changes occurring to the root cell walls.

Sections produced by microtomes, i.e. hand microtome, freezing microtome, sliding microtome, rotary microtome or ultramicrotome, all facilitate researchers in detailed inspection of internal structure of the plants. Though tedious and time consuming, microtome sectioning provides thin sections of the plant tissues or organs, perfect for histological studies after being properly stained through suitable staining procedures. The classical paraffin embedding methods of plant microtechnique were proposed way back to 1940 by Johansen and 1966 by Sass. Those techniques are still in fashion with some modifications by individual researchers to suit their objectives (Keating, 1996; Kermanee, 2008).

Among progressive steps of the whole procedure, staining has been most adjusted due to the chemistry of specimen tissues versus particular stains (Yeung, 1998). Fortunately, the number of dyes available is enormous, as well as the ways of employing stains, single or combinations alike, convenient for the workers to conduct their works. The well-known dyes used by botanical technicians are acid fuchsin, aniline blue, brazilin, carmine, crystal violet, erythrosin, fast green, hematoxylin, methylene blue, safranin and sudan (Johansen, 1940; Kermanee, 2008; Ruzin, 1999). Each dye has specific physical and chemical properties thus can react differently with particular structures. For example, hematoxylin appears excellent bright-field stain for nucleic acids, nucleus or chromosomes while safranin O performs bright red in chromosomes, nuclei and lignified, suberized, or cutinized cell walls. Fast Green gives perfect green in cytoplasm and cellulosic cell walls but turns blue in basic solutions and blue to bluish-green in stems and leaves of aquatic plants and most gymnosperms (Johansen, 1940; Ruzin, 1999).

Microscopic images of plant sections can be enhanced by TEM and SEM with higher and more powerful magnifications (Cutler *et al.*, 2008). Zaccai and Edri (2002), successfully followed on floral transition in lisianthus (*Eustoma grandiflorum*) with their SEM operating skills while Motum and Goodwin (1987) observed floral initiation of kangaroo paw (*Anigozanthos* spp.) by similar technique. Baksh and Richards (2006) examined morphology and anatomy of *Eleocharis*

cellulosa Torr. via SEM and described its shoot growth and branching patterns, capable of providing a model for the architecture of a spikerush species.

Several authors contributed their works on anatomical characters of Amaryllidaceae. Vitayasak (1996), in Thailand, working on 5 cultivars of *Hippeastrum*, a local red-flowered and 4 introduced cultivars, namely Apple Blossom, Orange Sovereign, Red Lion and Telstar, reported the structures of root, stem, leaf and flower of the plants. The root section revealed, inwardly, epidermis, cortex, endodermis, pericycle and stele. A transverse section of stem showed a single-layered epidermis with the cortex occupying most of the ground tissue, leaving the small part of the innermost being that of the stele. The leaf section consisted of upper and lower epidermis with mesophyll tissue in between. The vascular bundles of the leaf were found scattered in the middle part of the mesophyll. A transverse section of a flower indicated the inwardly floral whorls of perianths, androecium and gynoecium, consecutively.

Galanthus is another Amaryllidaceae attracting various researchers. Sahin (1998) studied morphological, anatomical and physiological nature of *Galanthus* collected from Turkey and produced the plants characterization records. Davis and Barnett (1997) accordingly described leaf anatomy of the genus from transverse sections, supplemented with epidermis characters, for the purpose of systematic identification. From the studies eight characters were claimed of having the potential to provide data for grouping of the species and subspecies. Some of these characters, alone or in combination with other anatomical data, were found capable of sorting out the relationships among taxonomically difficult species. The results of these studies were supported by classifications provided by Artjushenko (1966), based upon anatomical and morphological information. Recently, Özdemir and Alçitepe (2010) investigating morphological and anatomical properties of *Galanthus gracilis* examined and demonstrated anatomy of the plant, especially those of the flowers. They then indicated that the character of the outer surface of the inner perianth segments, having green patches at the apex and at the base, can be used as species identity.

Shou-Biao *et al.* (2006) studying comparative anatomy of the leaves of 12 species of *Lycoris* stated similarities of foliar anatomical characters among the species. But, some certain differences existed, providing valuable information of interspecific relationship and relevant utilization potentials of the plants.

Sultan *et al.* (2010) investingating anatomical properties of the leaves and roots of a bulbous plant, *Pancratium*, revealed the information obtained from botanical studies of the plants and their phytochemical information, valuable for further investigation towards potential discoveries of new natural bioactive compounds.

Meriç and Hüseyinova (2000) observed calcium oxalate raphide crystals of the adventive root tips of *Sternbergia lutea* (L.) Ker-Gawl. ex Sprengel, describing the ultrastructures of the crystal idioblasts in the cells of root tips and those of comparative meristematic cells. Peruzzi *et al.*, later in 2008, studying morpho-anatomical and karyologial of the genus agreed that morpho-anatomical features of the leaves, especially the leaf colour, leaf shape, leaf width and morphology of the median adaxial keel were of good taxonomic markers among the species. Karyological analysis revealed the chromosome complements of the genus *Sternbergia* as follows: *S. colchiciflora* 2n = 20; *S. lutea* 2n = 22; *S.sicula* 2n = 22. Nevertheless, the authors indicated that *S. colchiciflora* was confirmed to be the most isolated species, while *S. lutea* and *S. sicula*, being recognizable on morphological grounds, appeared karyologically very close to each other.

4.4 Chromosome investigation

Cytogenetics is a hybrid science that combines cytology, i.e. the study of chromosomes and other cell components, to genetics, which is the study of inheritance. This subject includes chromosome handlings, functions and movement of chromosomes, numbers and structure of chromosomes, numerous modifications of structure and behavior relating to recombination, and expression of genes (Singh, 1993).

4.4.1 Applications of cytogenetics

According to Jones and Luchsinger (1979) and Soontornchainaksaeng (2005), cytogenetical investigations are used to determine taxonomic botany and breeding research. As for taxonomic botany, chromosome numbers are generally used in classification of plant species. The DNA information of each species is organized in a characteristic number of chromosomes which is a reasonable indicator of relatedness among similar species. Variations in chromosome number affects the expression characters and behavior of plants which influence the variability of the species and

varieties (Kaewduangta, 2008; Poruksa, 1997). In breeding science, abnormalities in chromosomes of the plants, resulting in variations of phenotypes as called mutation, contributes tremendously to horticultural ornamentals, especially those of polyploids (Chaiyasut, 1989), as can be observed from the releases of improved polyploid and aneuploid varieties of commercial *Hippeastrum* (Patanakanog, 2001; Traub, 1958; Vitayasak, 1996).

Soontornchainaksaeng (2005) acknowledged advantages of cytogenetical investigation in plants, benefiting other scientific subjects of 1) plant systematics, since chromosome numbers can be used as a basis for classification of species, genera and families, 2) plant breeding, in terms of genotypic/phenotypic relationship determination, 3) plant genetic conservation, 4) phylogenetics, 5) molecular genetics and cell biology and 6) environmental monitoring, through investigation of chromosome aberrations of plants in toxified and contaminated environments.

4.4.2 Chromosome techniques

Number and type of plant chromosomes can be determined from various meristematic tissues at metaphase stage of mitosis or meiosis. Appropriate preparation of meristematic tissues can lead to accurate chromosome counts and karyotypic recording. Usually, the tissues taken for somatic chromosome investigation are those from the root tip, base of the calyx, young bud, the tip of young leaf, endosperm inside the seeds, where mitotic dividing cells occur (Apisitwanich and Masuthon, 2000; Chaiyasut, 1989; Jones and Luchsinger, 1979; Withner, 1974). Accordingly, reproductive tissues bearing microspore mother cells and megaspore mother cells are for meiotic studies (Apisitwanich and Masuthon, 2000; Baimai, 1993; Bootrat, 1985; Campiranon, 1997).

Cytologists have been devising cytological techniques in obtaining precise information on chromosome numbers and chromosome structures to examine cell mechanisms and to analyse genome and chromosome aberrations in plant species (Soontornchainaksaeng, 2005). Squash and smear techniques were found to be the basic methods of handling mitotic and meiotic chromosomes of plant species along with karyotype analysis and meiotic configuration (Campiranon, 2003; Soontornchainaksaeng, 2005). Among chromosomal techniques, the Feulgen's squash is the most popular for

karyotypic studies since it gives flattened chromosomes aligning at the same level, easier for inspection (Campiranon, 2003; Dyer, 1979; Krasaechai, 1996a).

Chromosome banding techniques were developed to facillitate identification of individual chromosome while Chromosome Image Analyzing System (CHIAS) has been used for karyotyping. Flow cytometry and chromosome sorting are used for relative measurements of DNA contents of nuclei, thus enabling measurement of ploidy and cell cycle including interspecific and intraspecific comparison of nuclear sizes. An advantage of this flow cytometry is the capacity of quantifying fluorescent objects in a population as they pass the light beam. Nevertheless, while molecular biologists rely tremendously on modern technologies of chromosome microdissection and *in situ* hybridization which are the technique for detecting specific nucleic acid sequence and for localizing highly repetitive DNA sequences in particular regions of the chromosomes with an aid of SEM, modest cytological researchers prefer to stick to the classic techniques like Fuelgen squash and smear methods. These methods requiring only simple instruments and chemical reagents but yet are adequate and competent in chromosome identification (Soontornchainaksaeng, 2005).

Dyer (1979), Sharma and Sharma (1980) and Singh (1993) similarly described the basic principles for handling mitotic and meiotic chromosomes of plant species as consisting of collection, fixation, maceration and staining of the specimens. Modification of the practice can be made depending upon crop species, objectives of the experiments and personal preference of individual technician.

Cytological research has been widely carried out in several plant species. *Hippeastrum* is one of those species with rather long evolution. Fernandez, since 1970, studied the chromosome of *H. bicolor* and *H. igneum* from the root tissue using squash method. Two techniques of pretreatments were used, one with saturated solution of para-dichlorobenzene and another with 1% colchicine. Fixation of alcohol and acetic acid (3:1) was applied and the tissue was stained with 2% aceto-orcein. The chromosome number of the two species were reported to be equal, i.e. 2n=16. Lakshmi, in 1980, studied chromosomal techniques in eight genera of Amaryllidaceae, using root-tip squash method. Pretreatments were done in saturated solutions of 1:1 para-dichlorobenzene and 0.002 M 8-hydroxyquinoline with the durations of 3-4 hours at the temperatures of 1-15°C. Fixation was followed in 1:3 acetic alcohol

mixture for 4-5 hours. The samples were then transferred to 70% alcohol and stored in refrigerator until further steps. The squash was done in aceto-orcein drops. In these studies meiosis were also carried out. Young flower buds of *Hippeastrum* and *Haemanthus* were taken from the bulbs for anther extraction. Detached anthers were then fixed in 1:3 propionic acid and alcohol for 24 hours then transferred to 70% alcohol. Staining and squashing were done via aceto-carmine smears. Williams and Dudley studied the chromosomes of *Hippeastrum iguazuanum* and reported in 1984 that the aceto-carmine squash technique was suitable and that the somatic chromosome count revealed 2n = 24.

Patanakanog (1999) worked on *Hippeastrum* species and some hybrids. The technique being used included the root tip sampling from the bulbs in aseptic culture during 8.30 to 9.30 a.m., pretreatment in a solution of 0.05% colchicine at 10°C for 20 hours, fixation in fixative solution for 24 hours, macerating in 1 N HCl at 60°C for 10 minutes and staining with 1% aceto-carmine for 12 hours. All specimens obtained the same chromosome number of 2n = 22. Apisitwanich and Masuthon (2000) reported their chromosome studies of *Hippeastrum*, *Eucrosia* and *Crinum* from root tip tissues using various techniques, a regular technique of aceto-orcein squash, Feulgen's squash with a modification of cell digestion in 2.0% cellulase RS, 0.3% pectolyase Y-23 and 1.5% macerozyme RA, then stained with giemsa dye. They found that cell-digesting technique yielded the best result where the cells spreaded out well, easy for counting. They reported the chromosome numbers of *H. reticulatum*, *C. asiaticum* and *E.* sp. being 2n=22, 22 and 28, respectively. Choticasatian (2002) experimented on root-tip squashes of 6 ecotypes of *H. johnsonii* Bury, i.e. the types with orange, pink, red and white flowers and 2 types of *H. reticulatum* var. *stratifolium* Herb., i.e. Waan-Rang-Nguan and Waan-Rang-Nak. She also studied the karyotype of *Hippeastrum* with white flowers by investigating from Feulgen's squash of the root tips. The technique was described in detail as 4 hours pretreatment in alpha-bromonapthalene at room temperature, fixation for 30 minutes in 90% acetic acid, macerating for 8-12 minutes in 1 N HCl at 60°C, staining in Schiff's reagent for 30-120 minutes and squashing in a drop of aceto-orcein. All of the samples showed 2n = 22.

Vitayasak (1996), Arayakitcharoenchai (2000), Sudsa-nguan (2001) and Chawalid (2003), all working on *Hippeastrum* chromosome investigations, agreed to the suitable technique of root tip preparation for somatic chromosome observation via squash method. The root tip samples were best taken during 9.30-10.00 a.m. The tissue was good when pretreated in PDB for 24-48 hours at 10°C prior to 5 minutes of fixation in fixative solution. Maceration should be done in HCl concentration of 1 Normal for 5 minutes at 60°C then stained in carbol fuchsin for 12-24 hours before squashing. They reported that this preparation technique provided a number of cells at metaphase stage with prominent chromosomes resulting in accurate chromosome counting and adequate karyotypic information.

Poggio *et al.*, in 2007, presented the karyotype and DNA content of 12 diploid species of *Hippeastrum* from South America. For squash preparations, root tips were pretreated for 2.5 hours in 0.002 M 8-hydroxyquinoline at 20°C, fixed in 3:1 of absolute ethanol and acetic acid, hydrolysed in 1 M HCl at 60°C for 10 minutes, and stained in Feulgen. In 2008, Sirikhum *et al.* (2008) reported their studies on chromosome investigation of small-flowered local *Hippeastrum* and large-flowered *Hippeastrum* hybrids. Chromosome numbers of 6 large-flowered varieties, i.e. Fanfare, Joker, Jungle Bells, Rozetta, Voodoo and Wedding Dance were investigated by means of Feulgen's squash technique. It was found, in all varieties tested, that the tissue from both root-tip and shoot-tip provided good results. Suitable sampling hours were 9.00 a.m. or 3.00 p.m. and pretreatment and staining durations were 48 hours and 24 hours, respectively. Macerating durations were best at 3 minutes for shoot-tip and 5 minutes for root-tip. The chromosome counts of the first five varieties were $2n = 33$ while the other was $2n = 44$.

As stated earlier, cytogenetical studies of *Hippeastrum* has a long history and abundant publications had been made. From such contributions a tabulated data of *Hippeastrum*'s chromosome counts was thus neatly produced as concluded in Table 1.

Table 1 Chromosome number of *Amaryllis* and *Hippeastrum*

Species/var./cv.	Chromosome number (2n)	Researcher and year of study
Amaryllis apertispatha	66	Traub, 1958
A. barreirasa	22	Traub, 1958
A. belladonna	22	Arroyo, 1982; Poggio *et al.*, 2007; Traub, 1958
A. blumenavia	77	Traub, 1958
A. calyptrata	22	Traub, 1958
A. elegans	22	Traub, 1958
A. evansiae	22	Traub, 1958
A. fosteri	22	Traub, 1958
A. maracasa	22	Traub, 1958
A. pardina	22	Traub, 1958
A. reginae	33	Traub, 1958
A. striata	44	Traub, 1958
A. stylosa	22	Traub, 1958
A. traubii	22	Traub, 1958
A. vittata	44, 43	Okubo, 1993; Traub, 1958
Hippeastrum advenum	18	Okubo, 1993
H. ambiguum	22	Okubo, 1993
H. argentinum	33	Okubo, 1993
H. aulicum	22	Arroyo,1982; Okubo, 1993
H. bicolor	16	Fernandez, 1970
H. blumenavia	22	Arroyo,1982
H. candidum	33	Okubo, 1993
H. cardenasianum	22	Meerow *et al.*, 1992
H. chiliense	18	Okubo, 1993
H. correiense	22	Poggio *et al.*, 2007
H. elegans	22	Okubo, 1993
H. equestre	22	Okubo, 1993; Soontornchainak-saeng and Chaiyasut, 1996; Sopinvetaya, 1980
H. evansiae	22	Poggio *et al.*, 2007
H. forgetii	22	Arroyo, 1982
H. igneum	16	Fernandez, 1970
H. iguazuanum	24	Williams and Dudley, 1984

Table 1 (continued)

Species/var./cv.	Chromosome number (2n)	Researcher and year of study
H. johnsonii Bury.	22	Chawalid , 2003; Choticasatian, 2002; Sudsa-nguan, 2001
H. lapacense	22	Meerow *et al.*, 1992
H. machupijchense	22	Poggio *et al.*, 2007
H. morelianum	22	Poggio *et al.*, 2007
H. parodii	22	Poggio *et al.*, 2007
H. pratense	18	Okubo, 1993
H. psittacinum	22	Poggio *et al.*, 2007
H. puniceum	22	Eksomtramage *et al.*, 2002; Okubo, 1993; Sudsa-nguan, 2001
H. reginae	33	Okubo, 1993
H. reticulatum	22	Lakshmi, 1980; Soontornchainak-saeng, 2005
H. reticulatum var. *striatifolium* Herb.	22	Chawalid, 2003; Choticasatian, 2002; Eksomtramage *et al.*, 2002
H. robustum	22	Okubo, 1993
H. solandriflorum	22	Okubo, 1993; Poggio *et al.*, 2007
H. spp.	22	Arroyo, 1982; Darlington and Wylie, 1955; Eksomtramage *et al.*, 2002
H. stylosum	22	Okubo, 1993
H. tucumanum	22	Poggio *et al.*, 2007
H. vittatum	43,44	Okubo, 1993
H. vittatum var. tweedianum	22	Meerow *et al.*, 1992
H. cv. Apple Blossom	44	Arayakitcharoenchai, 2000; Khaleel and Siemsen, 1989; Vitayasak, 1996
H. cv. Bahia ^{PPAF}	33	Meerow, 2000
H. cv. Basuto	44	Khaleel and Siemsen, 1989
H. cv. Bold Leader	44	Khaleel *et al.*, 1991
H. cv. Cocktail	44	Khaleel *et al.*, 1991
H. cv. Dawn	45	Karihaloo, 1985
H. cv. Desert Dawn	44	Khaleel *et al.*, 1991

Table 1 (continued)

Species/var./cv.	Chromosome number (2n)	Researcher and years of study
***H.* cv. Dutch Belle**	**44**	**Khaleel *et al.*, 1991**
***H.* cv. Fanfare**	**33**	**Sirikhum *et al.*, 2008**
***H.* cv. Intokazi**	**44**	**Khaleel *et al.*, 1991**
***H.* cv. Joker**	**33**	**Sirikhum *et al.*, 2008**
***H.* cv. Jungle Bells**	**33**	**Sirikhum *et al.*, 2008**
***H.* cv. Lucky Strike**	**44**	**Khaleel and Siemsen, 1989;**
***H.* cv. Miracle**	**44**	**Khaleel *et al.*, 1991**
***H.* cv. Orange Sovereign**	**44**	**Arayakitcharoenchai 2000; Vitayasak, 1996**
***H.* cv. Red Lion**	**43**	**Vitayasak, 1996**
***H.* cv. Red Strike**	**44**	**Khaleel and Siemsen, 1989**
***H.* cv. Rio** [PPAF]	**33**	**Meerow, 2000**
***H.* cv. Rozetta**	**33**	**Sirikhum *et al.*, 2008**
***H.* cv. Sampa** [PPAF]	**33**	**Meerow, 2000**
***H.* cv. Tangerine**	**44**	**Khaleel *et al.*, 1991**
***H.* cv. Telstar**	**44**	**Vitayasak, 1996**
***H.* cv. Voodoo**	**33**	**Sirikhum *et al.*, 2008**
***H.* cv. Wedding Dance**	**44**	**Sirikhum *et al.*, 2008**
***H.* cv. Zanzibar**	**44**	**Khaleel *et al.*, 1991**

5. Floral development of flowerbulbs

According to several authors, flowering process of flowerbulbs occur in similar manner as regular flowering plants. Nevertheless, particular flowerbulbs show responses to thermo- and/or photo-periodicities towards certain stages of floral initiation and development, making the process more complicated than other flowering plants (Suwanthada, 1990).

5.1 Floral initiation and development

Generally, when a plant reaches its reproductive stage of development flower formation may arise at the apex of the main shoot or at those of the lateral branches, or in both places (Esau, 1953; Fahn, 1974). Le Nard and De Hertogh (1993)

stated that the flowering process involves five successive stages of 1) induction, 2) initiation, 3) organogenesis, i.e. differentiation of floral parts, 4) maturation, i.e. growth of the floral parts and 5) anthesis. Evans (1993) indicated important aspects of flowering process being 1) the signals received by the plant instigate the process, 2) the transport to the shoot apex and 3) the changes occurring in the shoot apex during floral differentiation.

Evans (1993) also cited that although successive steps following floral initiation were more or less simple to recognize but the knowledge of the factors controlling them including determination of the period of the growth cycle taking place in the bulb were undoubltedly essential.

Studies on flowering in bulbous plants have been of tremendous interests, dated back as early as 1931 when Blaaum (cited in Paula, 2006) described the general flowering process in true bulbs. According to his analysis, bulbs underwent the following stages of I) the meristem was vegetative and produced leaves, II) the last leaf and new growing point were formed, III) a certain number of leaves were formed, and IV) the flower/inflorescence meristem was formed, followed by formation of flower (s) and its/their floral parts. His findings were recently supported by the work of De Munk and Van der Hulst (cited in Theron and De Hertogh, 2001) describing the flowering process as: Stage I: vegetative, Stage II: formation of spathe, Stage III: beginning of flower initiation, Stage IV: flower development and anthesis and Stage V: flower senescence and vegetative growth.

Kosugi (1942) revealed the sequence of events during floral initiation and development of *Lilium longiflorum* as I: vegetative, II: dome formation, III: floret primordium formation, IV: outer perianth primordium formation, V: inner perianth primordium formation, VI: stamen primordium formation, VII: pistil primordium formation, VIII: pollen tetrad formation and IX: flowering.

Salisbury later published his work in 1966 indicating his classification of flowerbulbs according to their behaviors of flower formation. He categorized the plants into 4 groups, referring to the timing of the flower formation, i.e. 1) the bulbs that flower formation occurred alternatively with leaf formation during the whole assimilation period, such as *Hippeastrum*, *Nerine* and *Narcissus*, 2) the bulbs that started their flower formation during the rest period, such as *Hyacinthus*, *Tulipa* and

Crocus, 3) the bulbs that their flower formation began during or towards the end of the storage period, but completed after planting, such as *Allium*, *Lilium* and *Galtonia* and 4) the bulbs that formed their flowers after replanting, such as *Gladiolus*, *Freesia* and *Anemone*.

In recent years, research involving the flowering process of flowerbulbs has been extensive and abundant works have been published. Vijverberg (1981) as later cited by Okubo (1993) working on flowering of *Hippeastrum* concluded the terminology of stages in flowering process occurring in the plant as follows: P1: initiation of outer petal, P2: initiation of inner petal, A1: initiation of outer stamen, A2: initiation of inner stamen, G: initiation of style and G+: advancement of style and occurrence of its lobes.

Vutthijumnonk (1990) investigating initiation and development of floral buds of *Eucrosia* sp. reported that initiation of the first floral bud was determined, based on the increase of the apex, by the first week of December. Development of the floral bud continued following by initiation of the others on the same umbel disc. The whole inflorescence took about seven weeks from the first floral bud initiation to completion of its development, resulting in a young inflorescence covered with the bracts, situated at the centre of the dormant bulb. This inflorescence then resumed its growth around the last week of March of the following year.

Pubuopiend (1992) studying flower development of a local curcuma, later identified as *Curcuma alismatifolia* Gagnep., described the sequence stages as I : vegetative stage, II : transition stage, Br : initiation of floral bract, Pr : initiation of the first floral primordium, D : division of the first floral primordium, P : initiation of petals, Sp : initiation of sepals, A : initiation of stamen and G : initiation of carpel. He also stated that the florets appearing in each coma bract, 6-7 in number, developed alternately in succession. Each of the florets obtained its own bracteole. Floret formation completed before spike emergence.

Rees (1992) studying intensively on the growth of bulbs stated the symbols indicating stages of flowering process of *Narcissus* as I, II, Sp, P1, P2, A1, A2, G and Pc while Le Nard and De Hertogh (1993) cited on those of *Tulipa* as I, II, P1, P2, A1, A2 and G and *Freesia* as I, II, Pr, Br, Bo, A, P1, P2 and G. The latter

authors also concluded in their text declaring the abbreviations, being widely used in describing the sequence of events in flowering process, as seen in table 2.

Table 2 Abbreviations used for stages of flower initiation and development in bulbs (Le Nard and De Hertogh, 1993)

Abbreviation symbol	Description of stage of development
I	Vegetative meristem: leaf forming stage
II	Transition to flower initiation: doming of meristem
Pr	First flower primordia visible (for bulbs with multiple flower)
Sp	Spathe stage, an enveloping sheath
Br	Flowers bearing bracts
Bo	Secondary bracts
P1	1st whorl of perianth
P2	2nd whorl of perianth
A1	1st set of androecium
A2	2nd set of androecium
G	Formation of gynoecium
Pc	Trumpet of *Narcissus* (paracorolla)

Suwanthada *et al.* (1997) studying flower formation of some flower-bulb species having different structure of bulbs summarized their work that the flowering patterns of the plants were different, relating to the bulb structure. It was stated from their findings that particular plant species, sharing the same structure of tunicate bulb could flower differently. Specific examples observed from their investigation were given, accondingly. *Eucrosia* sp., *Eurycles* sp. and *Haemanthus* sp. were similar in flower formation and flowering, each producing a single floral bud from the apical meristem inside the bulb. This floral bud resumed its growth prior to those of vegetative buds after the dormant period was broken. But, *Hippeastrum* spp. and *Zephyranthes* sp. behaved differently. For *Hippeastrum*, the floral buds appeared at every forth scale axil inside the bulb. The topmost floral bud, which was the first to bloom, located next to the apical vegetative bud. In case of *Zephyranthes*, the floral

buds, though laterally oriented inside the bulb, functioned sympodially. It was also stated here that some monocot rhizomes, such as *Curcuma*, flowered differently within species.

Hannantavivat (1998), following development of floral parts of *Eucrosia* sp. found that inside the immature florets of 0.3-0.5 cm in length the outer whorl perianth were already formed with those of the inner whorls in progress. The florets, 0.7-0.9 cm in length, developing inside the big bulb obtained anthers accommodating pollens in their prime age, elongated style and the ovaries with fully developed ovules.

Samatthiya reported her work on flower formation of *Haemanthus* sp. in 2000. Inspecting the growth of the bulb, she found that floral initiation occurred at a certain stage of vegetative growth. This initiation was followed by differentiation of the floral parts of several floret buds. Floret formation on the umbel disc was gradual, coincided with the entering of dormancy of the bulb. The sequence of a floret development was I, II, Pr, Br, P, A and G.

Arayakitcharoenchai and Suwanthada (2001b) followed floral development of 3 varieties of *Hippeastrum*, i.e., local red small-flowered, and two commercial large-flowered, Apple Blossom and Orange Sovereign. They described that initiation and development of inflorescences occurred similarly in all varieties. Floret development was closely observed by extracting florets of different size and age to examine their development, individually. It was found that floral development followed the pattern of P1, P2, A1, A2, G and G+. Special intention was paid on development of reproductive organs, beneficial for research works on improvement of the plant. Histological study of the florets, 1.5-2.2 cm in length, showed enlarged anthers attached to slightly elongated filaments. Meiosis of pollen mother cells occurred inside the pollen sacs of those anthers. Amphitropous ovules appearing in the ovary of the same floret performed axile placentation in doubled-row pattern in each of the three locules. Fully developed embryo sacs were readily found.

Puangkaew (2001) investigated floral growth and development of Brisbane Lily (*Eurycles amboinensis* Lindl.) and found that floral initiation took place at the growing point of the bulb as early as the completion of leaf abscission. Floral development continued until the bulb was through with its dormant period. Immature

inflorescence surpassed vegetative bud in growth, emerged and bloomed in the early stage of the new cycle of annual growth. Stages of floral development were recorded in sequence as I, II, Sp, Pr, Br, P, A and G.

Fukai and Goi (2001) revealed morphological changes occurring in the shoot apex of *Lilium longiflorum* Thunb. cv. Hinomoto that floral initiation appearred at the axillary buds. Once such initiations occurred the shoot apex produced a few number of leaves, then disintegrated. Each axillary bud consecutively developed into a floret, resulting in an immature inflorescence. Fleret formation took place at the swollen apex of the axillary bud, producing a bract and, later, the succession of three outer perianths, three inner perianths, six stamens, and, a pistil.

Buathong (2008) reported floral initiation and development of stamen and pistil of *Zephyranthes candida* Herb. that each flower bud developed from a lateral vegetative bud and that more than one flower buds were formed in each bulb in sympodial manner. Differentiation of the floral parts was in a sequence of I, II, Br, P, A, G. Development of stamen and pistil could be seen in each flower from the early stage of the floral development, when the folwer was still very small. Pollens and ovules were fully developed when the flowers were in their tight bud stage.

Fukai (2009) determining morphological development of inflorescence and floral organs of *Tricyrtis hybrida* reported that the plant obtained heteromorphous compound inflorescence, i.e. the main axis was indeterminate and the secondary axes were determinate. The apex of the main axis produced primary bracts continuously. The growing point appearing at each axil of those bracts produced a secondary bract then developed itself into a floret. Consequently, secondary florets were formed, following the same manner of the primary forets, i.e. the growing point situated at the axil of the secondary bract produced a tertiary bract then turned itself into the secondary floret. Tertiary and higher order bracts and florets were then formed, in similar pattern, leading to formation of a cincinnus. Each floret consisted of three outer and three inner perianths, six stamens, and a pistil, similar to other plants of Liliaceae. The basal part of outer perianths swelled and, later on, developed into spurs.

5.2 Factors affecting floral initiation and differentiation

It is well-known among plant scientists that flowering process of the plants are generally controlled by several internal and environmental factors. As for flowerbulbs one of the internal factors affecting most species is the age factor, so called juvinility.

Juvinility has been recognized by scientists and bulb growers alike as the limiting factor affecting flowering capacity of most bulb species. The bulb having the size less than its minimum is incapable of flowering (Rees, 1972). Hence, commercial value of the flowerbulbs is largely based on the bulb size (Hartmann and Kester, 1968).

According to Le Nard and De Hertogh (1993), the critical size for the flowering of bulbous plants are different, depending upon the plant species and specific varieties. For example, the critical size for tulip bulbs is between 6 and 9 cm in circumference, with some varietal differences, whilst that for hyacinth is between 6 and 8 cm in circumference. As for Imperator, Wedgwood and H.C. van Vliet irises, Rees (1972) indicated the minimum size to be 5-6 cm, 7-8 cm and 5-6 cm in circumference, respectively.

Okubo (1993) and Vijverberg (1981) stated the minimum bulb size of *Hippeastrum* being 20-24 cm in circumference, or larger. It was also said that for a plant to flower, the bulb needed to obtain nine leaves at the minimum. Bulb grades for commercial uses were agreed to be 20/22, 22/24, 26/28, 30/32 and 32/up in circumference (Okubo, 1993).

Flowering size bulb of *Eucrosia* were said to be 10.7-12.5 cm in circumference and 21-27 g in weight (Roh and Meerow, 1992). Phetchaburee (1994) studying flowering of this plant reported the better quality of inflorescences from larger bulbs over those from small bulbs. The bulbs reaching 100% flowering were those of 13.1-15 cm in circumference. Surapornpiboon (1996) working on similar subject supported his findings and reported that the bulbs smaller than 3.1 cm in diameter failed to form flowers.

Ahmad *et al.* (2009) observing tuberose reported that large bulbs, 3-4 cm in diameter, gave vigorous growth, maximum yield and more number of bulbils as compared to the bulbs of smaller sizes.

Referring to Le Nard and De Hertogh (1993) and Rees (1972), several naturally-occuring environmental factors can affect floral initiation and subsequent flower development of flowerbulbs. The major factors are temperature, light and moisture. These factors have been the subject of a large volume of work, and a number of reviews.

Okubo (1993) mentioned that *Hippeastrum* did not require low temperatures or specific day length for flower initiation. Such initiation occurred alternatively with the formation of four leaves without any interruption in growth. Accordingly, Door- duin and Verkerke (2002), investigating bud development and flowering of *Hippeastrum* under 15-25°C, observed at higher temperatures that larger bulbs produced more leaves whilst dry matter percentage of the bulbs decreased.

Theron and De Hortogh (2001) referred that flower formation did not occur in *Amaryllis belladonna* at 9, 13 nor 31°C, and that the optimal temperature for flower initiation of this species appeared to be 17°C. Higher temperatures reduced flowering percentage and flower quality.

Flower bud initiation and development of *Eucrosia bicolor* was studied and it was found that the bulbs stored at 10°C for 2 months remained vegetative, while those stored at 20°C reached a three-chambered gynoecium stage. Bulbs stored at 20°C flowered in 60 days while those stored at 10°C produced emerging leaves (Roh *et al.*, 1992).

Flowering process of bulbous plants can be controlled by applying specific treatments, e.g. temperature, moisture, light and plant growth regulators to the bulbs. However, to control flowering, precise knowledge of bulb periodicity is certainly essential (Bryan, 1989). In addition, sufficient information on the initiation and differentiation of the flowers is needed in commercial flower forcing to minimize the risk of flower abortion when the bulbs are forced (Slabbert, 1997).

6. Pollen physiology

Succesful pollination and fertilization are essential for sexual reproduction in higher plants. Pollen physiology has attracted the attention of plant breeders and horticulturists since the subject involves various aspects of studies related to

pollination, i.e. chemistry, storage, viability and germination of pollen, all applicable for plant improvement programmes (Agashe and Caulton, 2009).

6.1 Pollen viability and germination ability

Dafni and Firmage (2000) and Krasaechai (1996a) recognized that assessment of pollen viability is critical for the studies in breeding science on pollination biology, monitoring pollen vigour during storage, genetics and pollen/stigma interaction, crop improvement and breeding programmes, gene bank maintenance, incompatibility and fertility studies, evaluation of pollen germinability after exposure to certain conditions and evaluation of dispersal and gene flow. Among these, pollen viability and pollen germinability are of much interest to plant breeders as the fundamental information, useful for their breeding research. In breeding programme of plants, pollen viability and germinability of the parent plants are basically tested. Several methods for these tests are available, depending upon the aim of studies. Krasaechai (1996b) suggested the method of Random Scattering for evaluation of germination rate and the method of Line Up for that of the pollen tube growth as the most applicable ones.

The rate of pollen germination depends on the age of the pollen, types of flowers and environmental status. Factors affecting the growth of pollen tubes are said to be the level and the composition of chemicals in the pollen, structure and chemical composition of pistil, number and type of pollen, temperature and humidity and physiological conditions of stigma (Ruksat, 1996). Beyhan and Serdar (2008) referred to pollen viability as a capacity of the pollen to live, grow, germinate or develop. But, viable pollen grains might not actually germinate if the conditions were not suitable. They also said that there were several methods used for estimation of pollen viability and germinability in horticultural crops, to match objectives of the studies. The most common methods were suggested by Agashe and Caulton (2009), Krasaechai (1996b), Patanakanog (1999), Ruksat (1996), Shivanna (2003) and Shivanna and Johri (1985). These methods are as follows:-

6.1.1 Non-vital stain test

Pollen staining test is simple, convenient, rapid, instant and easy to practice, though the colour of the pollen is usually less accurate, due to the enzymes

appeared in the pollen of different maturation. Quite a number of stains can be used in the non-vital staining tests, such as Alexander's stain, aceto-carmine, acid fuchsin, aniline blue in lactophenol, FCR, FDA, iodine in potassium iodide, MTT, TTC and X-Gal. These stains react with active oxidatives or peroxidase enzymes present in cytoplasm of the pollen, giving rise to the red colour, suggesting the viability of the tested pollens (Beyhan and Serdar, 2008). Karihaloo (1989) working on *Hippeastrum* pollen tests, reported the results of the tests of aceto-carmine stain on 18 varieties of the plant, showing high variation of the pollen viability, ranging from 13.3 to 94.7%.

6.1.2 Tetrazolium test

Triphenyl tetrazolium chloride is widely used to test pollen viability. This stain reacts well with viable pollen and gives red colouration. The only limitation of the test using this stain is that the colouration of responding pollen shows a gradation from very light to dark red with the result that the cut-off point for scoring viable pollen cannot be very subjective (Shivanna, 2003)

6.1.3 *In vitro* germination test

The most commonly used and acceptable test for assessing pollen viability is believed to be the *in vitro* germination test. It is rapid, reasonably, simple while the results of *in vitro* germination generally correlate with seed set data. However, a major limitation of this test is the lack of optimal germination medium for pollen of many species.

Medium culture used for *in vitro* germination test can be that of liquid or agar. Many pollen grains can germinate in water or aqueous solutions of sucrose with no additives. However, the pollen of some species needs special substrates for germination (Beyhan and Serdar, 2008). Bootrat (1985) reported that a medium containing 0.5 g agar in 25 ml distilled water with 1 g glucose and 0.5 g gelatin was suitable for many kinds of flowers while Ruksat (1996) reported that pollens could also germinate well in a medium culture containing 1.5 g gelatin in 100 ml water with 16 g glucose, adjusting the pH to neutral with KOH or HCl. Stepka *et al.* (2000) mentioned that pollen germination of *Ornithogalum virens* L. cultured in liquid medium were higher than in agar medium.

Krasaechai (1996a) described the concentration of sugar in the medium as an important factor influencing germination rate of the pollen grains, with

appropriate concentrations depending on the plants. Too low concentrations can break the pollen tube while too high can cause abnormal or retared growth of the pollens. Pretests of pollen germination in a series of sucrose solution, usually between 0-50%, could serve to evaluate the osmotic relation of the pollen grains, well enough to disclose the optimal concentration for germinability tests for specific species. Beyhan and Serdar (2008) indicated that composition of germinating medium could dramatically affect pollen metabolism. Sharma *et al.* (1982) testing pollen germination of *Amaryllis vittata* in a liquid medium containing 3% sucrose with 2% pentaerythriol in the dark reported that such medium was appropriate for pollen germination. Arayakitcharoenchai and Suwanthada (2010) and Chawalid (2003) detecting pollen viability of *Hippeastrum* by culturing the pollens in liquid medium containing 3% sugar reported that the pollen grains germinated with relatively high percentages. Agashe and Caulton (2009) mentioned that bacteria or other microorganisms could contaminate the culture medium containing sugar. Cautions were suggested to avoid such contamination.

Thind *et al.* (1996) regarded nutrients, vitamins and growth regulators as factors capable of promoting or inhibiting the growth of pollen tube. From their studies, they found increases of IAA and GA_3 vs decreases of BA and ABA in microspores of *Amaryllis vittata*, while germinating. When these growth promoters, i.e. IAA and GA_3, were added in the medium, it occurred that they could enhance the growth of the pollens. Accordingly, Bhandal and Bala (1991), Sidhu *et al.* (1986) and Thind and Malik (1996), studied in the same plant reported reduced pollen germination rate when proline was added to the medium. Heavy metals such as Co, Ni, Pb and Zn could also inhibit germination of the pollen.

6.1.4 Fluorescein diacetate (FDA) test

Advanced techniques using fluorescence dyes allow observation of the pollen under fluorescence microscopy. This method has been the most commonly used in recent years. The FDA test has proven satisfactory in assessing pollen viability in a number of species, giving better index of viability than *in vitro* germination.

6.1.5 Fruit and seed-set

This method is agreed to be the most authentic and accurate test. Viability of the pollen is expressed through the ability of the pollen to effect

fertilization, resulting in seed and fruit set. But, it is also agreed that this method of testing has many limitations as laborious and time consuming.

6.1.6 Pollen tube growth in pistil test

The method of detecting pollen tube growth in the style attached to the pollinated stigma, though markedly reduces the time taken comparing to the fruit and seed set method, is not always feasible to quantify the number of pollen tubes growing in the style.

6.2 Pollen storage

Agashe and Caulton (2009) regarded pollen storage as essential in plant breeding. Successful pollen storage is a very convenient tool in the hands of plant breeders for improving plants by hybridization, especially under physical and/or environmental situations inappropriate for crossings. Suitable methods of pollen storage, depending upon the plant species, can prolong longevity of pollen grains with good quality close to those of the fresh ones.

Ruksat (1996) cited that although pollen storage can assist the plant breeders in their works but its effectiveness relies abundantly on the methods being used as well as the response of individual species. It was stated that pollen of some flowers could be kept for 1-2 hours at room temperature and the viability deminished within 1-2 days. Pollen could be stored for a long period of time in closed containers in a vacuum under dark with 5-10% humidity.

Agashe and Caulton (2009), Faegri and Iversen (1989) and Shivanna and Johri (1985) described that pollen storage could be done in several ways, such as storing under controlled temperature and humidity, freeze storage (Lyophilization), dry store at 10°C, straight deep-freezing, storing under controlled gases and pressure, storing in organic solvents and storing in pollen diluents. However, each method had varied limitation.

Several researchers had been successful with pollen storage of flowerbulbs. Shivanna and Johri (1985) succeeded in storing the pollen of *Lilium longiflorum* using various methods. They announced that the pollen stored for 50 days at 25°C produced the tubes of 5.6 mm long while those stored for 100 days failed to produce any tubes. They also found that the pollen grains stored initially for 50 days

at 25°C then shifted to acetone for up to 70 days produced pollen tubes of 5.8 mm long while the fresh pollen grains produced 8 mm pollen tubes in 24 hours at 28°C.

Bowes (1990) reported 2 methods of long-term storage for *Narcissus* pollen, 1) storing anthers in glass vials held in a desiccator containing calcium chloride at 2°C and 2) storing anthers or naked pollens immersing in liquid nitrogen in polypropylene straws. Pollens from both storage treatments showed 15-16% *in vitro* germination after 3 days, compared with 27.4% of the fresh pollens. The pod developed from the flower fertilized with pollens stored for 3 days was comparable to that from fresh pollens in term of seed setting. However, after 351 days, pollen from the anthers stored at 2°C exhibited only 0.1% germination and failed to set fruit after fertilization whereas no changes in germination rate were recorded from the pollens of the two liquid nitrogen treatments. Fruit set performances obtained from the treatments of stored pollens were equivalent to those from the fresh pollens.

Pfeiffer (1936) reported successful storage of *Hippeastrum* pollen by manipulation of temperature and humidity. The results revealed with *Amaryllis* experiment that stored pollen of *A. hybrid* gave better results when kept for one year at 10°C. In addition, Patanakanog (1999) stated the results of her experiment on the storage of local *Hippeastrum* pollen at 4°C that fresh pollen of the 4 varieties tested gave relatively high germination at 34.18% and decreased to 1.36% after 6 weeks. Subsequently, Arayakitcharoenchai (2000) and Chawalid (2003) worked on pollen storage of particular *Hippeastrum* belonging to different groups of diploids and tetraploids reported that pollen of the plants could be successfully stored dry at 5°C for the periods of up to 3-4 weeks with 60-75 germination percentages. Germination rates then declined with time and terminated within 12-15 week, depending upon varieties.

7. Seed and embryo development

Bewley and Black (1943) noted that the new plant formed by sexual reproduction started as an embryo within developing seed, which arose from the ovule. When mature, the seed was the means by which the new individual was dispersed. The success with which this new individual was established was largely determined by physiological and biochemical features of the seed.

Achievements in interspecific and intergeneric hybridizations, particularly among the parent plants of different ploidy, rely tremendously on vitality of the fertilized seeds to reach their maturity. On this expense, knowledge on seed development including that on embryology has been a great attraction to plant breeders.

7.1 Seeds

Seeds of flowerbulbs are different in size, depending upon species. They are also different in nature, some obtain dormant period while others germinate very quickly. Seeds of species belonging to Amaryllidaceae having similarities of large sized, round-shaped and high in water content (Rees, 1972). Traub (1958) as well as Rees (1972) indicated *Hippeastrum* seeds of having the same properties. The seeds lost thair viability during storage. The best germination was obtained immediately after harvest. Akavipat and Maneerat (1979) and Pindel (1990) said that storing the seeds at suitable temperature and humidity could prolong viability and capability of germination quite effectively. Germination rate of the seeds was highest at constant 25°C storage but decreased when stored above 25°C or below 20°C, indicating a narrow temperature range for maximum seed germination. Seed storage at 11% or 52% relative humidity, at 5°C or 15°C was proved effective in keeping the seeds up to 12 months with good viability (Carpenter and Ostmark, 1988a, b). In addition, Amico Roxas *et al.* (1994) found that 30 or 60 days of cold storage at 4°C had no negative effects on seed viability. Besides, cold storage was able to promote seed germination and shoot emergence. Positive correlation was found between seed size and seed viability. Seeds of large size produced bigger bulblets of higher weight than those of the small size.

Arayakitcharoenchai (2000) and Sudsa-nguan (2001) observed that fresh *Hippeastrum* seeds germinated within 14 to 30 days, with very high germinating percentages but, on the contrary, first emergent leaflet grew very slow. Seedlings needed to be fed regularly with fertilizers. Transplanting could enhance the growth of the seedlings to a certain extent. Plants from seedlings developed to their flowering stage in one and a half to two years. However, according to Okubo (1993), the exact flower- ing size of the bulbs depended on selection and environment conditions.

7.2 Embryo development

Stern *et al.* (2003) cited that embryo was an immature sporophyte that developed from a zygote within an ovule after fertilization. Esau (1977) stated that an embryo of flowering plants consisted of cotyledon, epicotyl and hypocotyl. These embryos varied in their relative volume and orientation in the seeds, which were the features that determine in part whether the embryo was upright, bent or curved. They also varied in degrees of development.

Development of an embryo is clearly described from fertilization that the zygote undergoes an asymmetrical cell division that gives rise to a small apical cell, which consequently becomes the embryo and the suspensor, having functions to provide nutrients from the endosperm to the growing embryo. From the eight cell stage onwards, zygotic embryo shows clear embryo patterning, which forms the main axis of polarity, and the linear formation of future structures. In globular stage, the embryo develops radial patterning through a series of cell divisions to produce cotyledon, hypocotyl, shoot and root meristem. Bilateral symmetry is apparent from the heart stage. In the subsequent torpedo and cotyledonary stages of embryogenesis, the embryo completes its growth by elongating and enlarging (Bewley and Black, 1943).

Differences between the organography of mature embryo of monocots and dicots are the cotyledon and the shoot apex. The embryo of most dicots obtains a pair of lateral cotyledons between the bases of which situated the rudimentary terminal shoot apex. In contrast, the typical embryo in monocots develops a single, terminal cotyledon, and the shoot apex appears lateral in position (Foster and Gifford, 1959).

Embryo development in flowerbulb has been a topic of interest among scientists. Brown *et al.* (1997) studied embryo development of *Nerine*. They stated from their investigation that at the time the seed was shed from the parent plant, approximately 5 weeks after pollination (AP), the embryo was then undeveloped, globose and approximately 100 μm in diameter. The majority of embryo development took place after the seed was shed. It became polar and underwent some external differentiation over the next five weeks. Rapid enlargement and elongation of the embryo did not start until 2-3 weeks prior to emergence from the seed, about 13 weeks AP. The embryo remained uncoiled and small, relatively to seed size,

occupying no more than 5% of the seed volume. The germinating embryo was positively geotropic and the tip underwent swelling to form a pro-bulb from which the contractile root emerged. The cotyledon became green but the tip remained inside the seed which did not degenerate until well after the emergence of the first true leaf, approximately 3 weeks after germination and almost five months post anthesis. Van Tuyl *et al.* (1989) reported his finding on *Lilium* that after fertilization, embryo abortion and/or endosperm degeneration could cause barriers in different stages of development. Using the embryo rescue technique, embryos could be saved by *in vitro* culture. Accordingly, embryo culture could be applied successfully in incompatible crosses, rescuing plants from inherently weak embryos, obtaining haploid plants as well as shortening the breeding cycle (Sharma *et al.*, 1996). This method has been applied in a large number of crops. Some recent examples were *Allium*, *Alstroemeria*, *Freesia*, *Lilium, Tulipa, Zantedeschia* (Van Tuyl, 1997) and *Hippeastrum* (Sandler-ziv *et al.*, 2004 cited in Benschop *et al.*, 2010).