PHYSIOLOGY AND MOLECULAR BIOLOGY OF ETHYLENE SYNTHESIS IN DENDROBIUM FLOWERS FOLLOWING COMPATIBLE AND INCOMPATIBLE POLLINATION

INTRODUCTION

Orchid is the most important cut flower of Thailand for export. The vase life of orchid flowers on the plant is usually very long. However, there are many causes to shorten flower longevity including harvesting, emasculation and pollination. Generally, pollination accelerates the senescence process more rapidly than emasculation and harvesting (Hew and Yong, 1997). Pollination affects floral longevity but this seem independent of its ability to increase ethylene production (Abeles *et al.*, 1992). Previous studies have suggested that ethylene plays a regulatory role in early senescence. The pollen of many flowers species contain 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene production in higher plants, and auxin (Reid *et al.*, 1984; Hill *et al.*, 1987). Furthermore, application of ACC and auxin on the stigma of carnation and orchid flowers resulted in symptoms similar to those observed after pollination (Reid *et al.*, 1984). Many studies have been carried out on the post-pollination events in many kinds of flowers (Pech *et al.*, 1987; Singh *et al.*, 1992; Porat et al., 1998; Ketsa and Rugkong, 2000).

Self-incompatibility (SI) is well known in term of genetical meanings. SI is a reproductive strategy of flowering plants that enables the pistil to reject geneticically related pollen. Incontrast, non-self pollen is accepted by the pistil and its tube grows down through the style to reach the ovary where fertilization takes place (McCubbin and Kao, 2000). SI was found in many kinds of plant such as petunia (Herrero and Dickson, 1979). For *Dendrobium* orchids, it rarely reported about the incompatiblility machanism. In this experiment, the definition of compatibility is an effect of pollination to induce early senescence and ovary growth of pollinated flowers whereas incompatibility is opposite effect of polliniated flowers. These studies have been performed and focused on interaction between pollen and stigma under

condition compatible and incompatible pollination in *Dendrobium* orchid-cut flowers. In these conditions, the objectives of this study were:

1. To compare the physiological changes and ethylene production following compatible and incompatible pollination of *Dendrobium* flowers.

2. To determine the activities of ethylene biosynthesis enzymes in pollinated *Dendrobium* flowers following compatible and incompatible pollination.

3. To determine the gene expression regulating enzymes in pollinated *Dendrobium* flowers following compatible and incompatible pollination.

LITERATURE REVIEWS

Orchid flowers

Orchids are monocotyledon plants that are members of the Orchidaceae family. This family is probably the largest in the plant kingdom with 750 different genera comprising at least 25,000 species and more than 30,000 cultivated hybrids. Shoots of orchid can grow according to two different patterns of growth: sympodial and monopodial. In case of sympodial growth, the elongation of the shoot is limited by the formation of flowers or inflorescences. Then, the plant develops through the formation of laterally located axillary buds. In opposition, the growth of monopodial orchid is continuous with unlimited activity of the apex. The orchid inflorescence consists in an axis that bears individual flowers along its length. The axis is divided into two regions: (i) the peduncle which is the axis region from the stem or base of pseudo bulb to the point of insertion for the flower, (ii) rachis, the remaining part of the axis containing the flowers. The oldest flower is located near the base of the axis and flowers are progressively younger along the axis towards the top of the inflorescence (Figure 1). Orchid flowers are zygomorphic (symmetrical about a single plane). Each flower has three sepals corresponding to the outermost part of a flower, and three petals. Two of the petals located in the lateral part of the flower are usually equal in size and shape, whereas the petal located in the lower parts of the flower has a different shape in form of a lip and known as the labellum (Figure 2). The labellum of many orchids is modified to form a spur, a cone like structure that protrudes towards the back of the flower, where nectar is produced (Hew and Yong, 1997). During development, the flowers turn upside down. This phenomenon is termed resupination (Arditti, 1992). The column is unique to orchids. It is a coalescence of both the male and female reproductive organs. The anther cap lies at the tip of the column and encloses the pollinarium with the rostellum underneath. The pollinarium consists of pollinia (masse of pollen), viscidium (a sticky disc) and stipe (thin strip of tissue connects the pollinia to the viscidium). Beneath the rostellum lies the stigma that is a cavity filled with sticky fluid. The stigma is connected to the ovary by the column that allows the growth of pollen tubes toward

the ovules during fertilization. The inferior ovary containing ovules is below the point of insertion for the sepals and petals (Hew and Yong, 1997).

Dendrobium orchid is the most important export cut-flowers of Thailand. It is the second largest genus after *Bulbophyllum* ranging through all parts of Asia and the Pacific and is the second most common orchid genera in cultivation after the *Cattleya*. It is found from eastern India through all of Asia and up into China and then out through all the Pacific islands including Australia to the south (Anonymous, 2005[1]). There are over 250,000 different orchid species and hybrids. The most decorative have been bred for cut flower use (Anonymous, 2005[2]). *Dendrobium* is epiphytes. Its growth pattern is sympodial. The pseudo bulbs, a well developed water storage organs, are called "canes" for their upright, leafy appearance.

Pollination and post-pollination events

Pollination is an important process which is required for the successful sexual reproduction in flowering plants. It induces early senescence in many flowers such as carnation, cyclamen, petunia, tobacco, and orchid (Halevy *et al*., 1984; Hill *et al*., 1987; Stead, 1992; Halevy, 1995[1]; Porat *et al*., 1995; Porat *et al*., 1998; Ketsa and Rugkong, 2000; Xu and Hanson, 2000; Ketsa, *et al*., 2001). Generally, the pollination process can be divided into three stages: 1) contact of pollen with the stigma, 2) passage of pollen tubes into style, and 3) fertilization of ovules (Halevy, 1995).

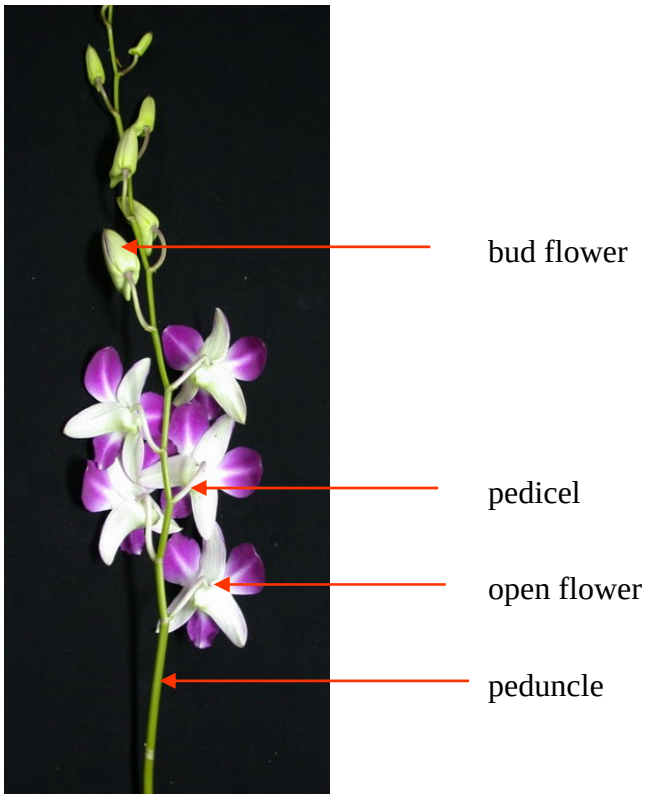

Figure 1 Inflorescence of *Dendrobium* orchid

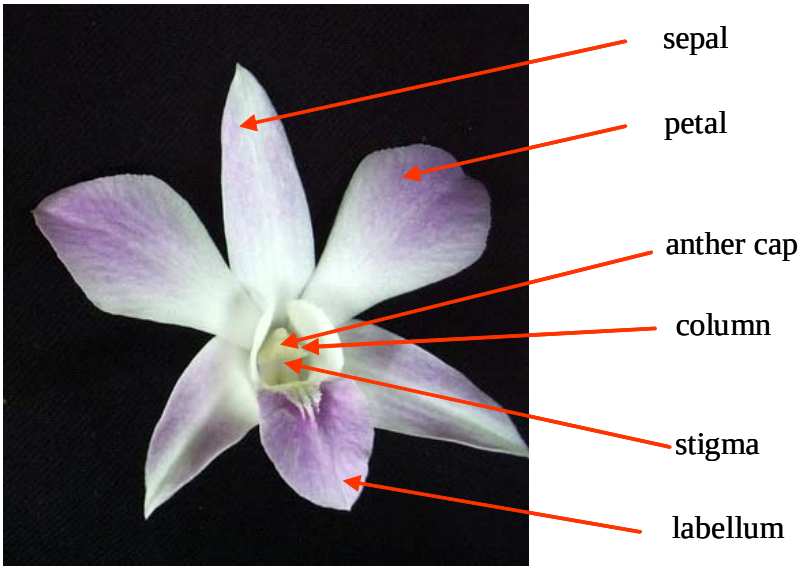

Figure 2 Parts of orchid flower

Pollination regulates developmental events and collectively prepares the flowers for fertilization and embryogenesis. Flower development can be divided into two stages. The first stage, prepollination, prepares the floral organ for pollen dispersal and reception. The second stage, postpollination, sheds some floral organs and prepares others for fertilization, embryogenesis and fruit development including suitable developmental changes. These changes consist of corolla senescence, pigmentation changes, ovary maturation, and, in some species, ovule differentiation and female gametophyte development (Zhang and O'Neill, 1993; O'Neill, 1997; O'Neill and Nadeau, 1997). The studies of pollination-induced ovule differentiation in the *Phalaenopsis* orchid flower have established the basic information of timing and hormonal stimuli to allow further characterization of gene expression associated with the timing of major developmental transitions during ovule development (Zhang and O'Neill, 1993).

In orchid flowers, pollination triggers the biochemical, metabolic and physiological changes in orchid flowers (Arditti, 1992; Avadhani *et al*., 1994; Hew and Yong, 1997). These changes are:

1. Increased respiration
2. Increased RNA synthesis or production of new RNA, or both
3. Production of new protein or increased synthesis of existing proteins, or both
4. Activation and / or synthesis of several enzymes, transport of organic and inorganic substances from perianth segments into the gynostermium and ovary
5. Anthocyanin synthesis or destruction
6. Chlorophyll synthesis or destruction
7. Cessation of scent production
8. Hydrolysis of the storage and structural molecules
9. Appearance of yellow pigments
10. Starch accumulation in ovary
11. Ethylene evolution

12. Swelling of ovary
13. Changes in pedicel curvature
14. De-resupination
15. Closure of stigma
16. Swelling and loss of curvature by the gynostemium
17. Ovule development
18. Senescence of some or all perianth parts
19. Re-differentiation of some floral segments
20. Nastic movements, such as hyponasty of sepals and petals

All these changes may occur for facilitating fruit set and development (Clark *et al*., 1997). However, among them the senescence of the perianth has considerable impact on the quality and shelf-life of flowers and therefore has received great attention from both the scientific and applied stand point.

Stead (1992) has discussed the twofold advantage for the flower to reduce longevity upon pollination. First, a shorter flower longevity would ensure that no excessive amount of pollen will be deposited upon the stigma for a full seed set. Furthermore, deposition of pollen is thought to be wasteful as growth of excessive pollen tubes competes for a limited pool of resources. Second, the maintenance of elaborate floral structures is a costly process in terms of water and energy. To achieve cost-effectiveness, the strategies have been developed by plants that include:

(1) Reduction or modification of nectar composition
(2) Structural modification (corolla wilting) and modification of color (fading)
(3) Abscission of all or part of corolla

Role of ethylene synthesis in the regulation of post-pollination events

Pollination not only accelerates early perianth senescence but also increases ethylene production (Porat *et al*., 1995; Woltering *et al*., 1995; Woodson and Lawton, 1988). Ethylene is a gaseous plant hormone and the simplest organic molecule with biological activity. It can be produced in all parts of higher plants,

however the rate of production depends on the type of tissue and the stage of development. Generally, meristematic regions and nodal regions are the most active in ethylene biosynthesis. Ethylene production is also increased during leaf abscission and flower senescence, as well as during fruit ripening (Have and Woltering, 1997). Auxin, wounding and physiological stresses such as flooding, chilling, diseases, and temperature or drought stress can induce ethylene biosynthesis (Woodson *et al*., 1992). Ethylene is released easily from the tissue and it diffuses in the gas phase through the intercellular spaces and outside the tissue. The ethylene biosynthetic pathway in pollinated flowers is the same as in ripening fruit and wound tissue. An amino acid, methionine, is the precursor in the ethylene biosynthesis pathway. The first step, SAM synthase catalyzes methionine to SAM (S-adenosyl methionine). After that, SAM is converted to ACC (1-aminocyclopropane -1-carboxylic acid) by ACC synthase. Finally, ACC is oxidized to ethylene by ACC oxidase (Yang and Hoffman, 1984; Taiz and Zeiger, 1998).

ACC is the immediate precursor of ethylene. Not all the ACC found in the tissue is converted to ethylene. ACC can also be converted to a nonvolatile conjugated form, *N*-malonyl-ACC (MACC) (Taiz and Zeiger, 1998). Malonylation of ACC to MACC deprives the ACC pool and reduces ethylene production (Kevin *et al*., 2002).

ACC synthase (ACS) is a key enzyme, which is considered as the rate-limiting step in ethylene biosynthesis, while ACC oxidase is considered to be constitutive in many tissues (Kende, 1993). ACS is involed in the conversion of SAM to ACC. It is a cytosolic enzyme with a very short half–life. Its activity is regulated by several environmental and internal factors, such as wounding, drought stress, flooding, and auxin. The regulation of ACS has been an intensively studied. Many ACS genes have been identified and cloned from different plant species, for example zucchini, tomato, winter squash, apple, carnation, mung bean and *Arabidopsis*. ACS is encoded by a multigene family whose members are differentially regulated (Taiz and Zeiger, 1998).

ACC oxidase (ACO) or ethylene-forming enzyme (EFE) is generally not the rate-limiting point in ethylene biosynthesis, although tissues that have high rates of ethylene production, such as ripening fruit and senescing flowers, show increased levels of ACO activity and mRNA. ACO is encoded by multigene families in several plant species (Johnson and Ecker, 1998). *ACO* gene is isolated and cloned from numerous plant species. The deduced amino acid sequences of *ACO* revealed that these enzymes belong to the Fe^{2+}/ascorbate oxidase superfamily. It is suggested that ACO might require Fe^{2+} and ascorbate for activity (Taiz and Zeiger, 1998).

Ethylene is known to play a key role in regulating the biochemical and anatomical changes that constitute the post-pollination syndrome. There are several explanations for ethylene increment after pollination. Increased ethylene production probably be inducted by ACC or auxin in the pollen. Other mechanisms include stigmatic recognition of pollen and tissue damage associated with stigma penetration and pollen tube growth. A study of ACC content of pollen from 15 species and their effect on petunia senescence suggested ACC content was not correlated with senescence (Abeles *et al*., 1992). However, the senescence of the cross-pollinated *Dendrobium* 'Pompadour' flowers was in good correlation with the ACC content of pollinia. High ACC content in pollinia promoted high rate of ethylene production and induced earlier senescence of cross-pollinated flowers as compared with cross-pollinated flowers by pollinia with a lower ACC content (Ketsa and Luangsuwalai, 1996).

Woodson *et al.* (1992) had examined the expression of mRNAs for S-adenosyl methionine synthetase, ACS and the ethylene-forming enzyme (EFE or ACO) in various floral organs of carnation during the increase of ethylene biosynthesis associated with petal senescence. The abundance of *ACS* and *ACO* mRNAs increased and S-adenosyl methionine synthetase transcripts decreased concomitantly with the ethylene climacteric in senescing petals. Furthermore, and increase in *ACS* and *ACO* transcripts was detected in petals from pre-senescent flowers within 3 to 6 h of exposure to 2 microliters per liter of ethylene.

Nadeau *et al*. (1993) had studied post-pollination changes of *Phalaenopsis* orchid floral tissue by RNA blot hybridization. It appeared that the increase in ACO activity was due to *de novo* synthesis after pollination of mRNA and presumably protein. Furthermore, the pattern of induction was consistent with a model of coordinated regulation of gene expression in which the pollination signal travels to other organs of the flower to induce their ethylene production. They used *in situ* hybridization to further define the temporal and spatial expression of *ACO* within the floral organs. The result showed the expression, and, by inference the capability to oxidize ACC to ethylene was induced in all living cells of the tissues examined after pollination. This finding contrasted with work in petunia that suggested that ACO was localized to the stigma surface (Pech *et al*., 1987).

Role of ethylene sensitivity in the regulation of post-pollination events

There are two reasons that pollination induces ethylene production and then flower senescence. One is to promote ethylene production and the other to increase the sensitivity of the flower to ethylene, thus cause them to respond to internal and external ethylene (Halevy *et al*., 1984; Halevy 1995[1]). The sensitivity factor was transmitted from gynoecium to the perianth lead to early senescence of perianth. Following pollination, some putative sensitivity factors were identified such as GTP-binding protein (Porat *et al*., 1994), auxin (Zhang and O'Neill, 1993), and short-chain fatty acids (Halevy *et al*., 1996). The most likely candidates are short-chain fatty acids (C_7 –C_{10}). The level of these acids, especially octanoic acid (C_8) in the perianth increased soon after pollination, and application of octanoic acid to the stigma of unpollinated *Phalaenopsis* flowers greatly increased the sensitivity of the flowers to ethylene. Application of octanoic acid increased the fluidity of gynoecium and perianth membranes indication an effect of this agent via membrane (Halevy, 1995). Whitehead and Halevy (1989) suggested that the synthesis of ethylene sensitivity factors, short chain saturated fatty acid increased ethylene sensitivity.

Role of signals other than ethylene. Interactions auxin/ethylene

The nature of the pollen-pistil interaction that leads to increase in ethylene biosynthesis was not clear (Larsen *et al*., 1995). However, pollination stimulates a dramatic changes in the metabolism and development of various flower parts (Halevy, 1995[2]). ACC and auxins have been proposed as the pollination signals. Pollen is rich of ACC and auxin. They have been reported in many kinds of plants including orchid pollinia (Arditti, 1979).

Application of auxin to the stigma of orchid led to increase in ethylene production and stimulated ovary development in a manner similar to pollination (Zhang and O'Neill, 1993). Auxin triggered ethylene biosynthesis. This phenomena was a common response of sensitive species. Auxins have been shown to induce *de novo* synthesis of ACS through increased expression of specific ACS genes or post-transcriptional regulation (Taiz and Zeiger, 1998; Hansen and Grossmann, 2000). Whereas application of ACC to the stigma just induced early perianth senescence.

Mól *et al.* (2004) reported that the ACC content of corn increased in all pistil parts soon after pollination. Besides the auxin level increased rapidly in the silks and ovaries after pollination, and it was also very high in the pollinated silks due to the high indole-3-acetic acid (IAA) content of pollen grains. The rise in IAA also appeared in the silks and ovaries after treatment with sand (mock pollination) but it was delayed by 8 h. Application of ACC (10 μM) or IAA (6 μM) solutions to non-pollinated silks stimulated maturation of the egg cells. Moreover, the response of the egg cells to pollination was canceled by L-α-(2-aminoethoxyvinyl)-glycine, α-aminoisobutyric acid or 2, 3, 5-triiodobenzoic acid applied to the silks before pollination. The authors (Mól *et al.*, 2004) concluded that ethylene synthesis and polar auxin transport in the silks pollinated with fresh pollen were necessary to evoke accelerated differentiation of the egg cells in maize ovules.