# CHAPTER 2
# LITERATURE REVIEW

## 2.1 ORCHIDS IN THE WORLD MARKET

### 2.1.1 The Economic Importance of Orchids

The popularity of orchids have transformed these plants from once being an exotic plant found only in the wild into one of the most demanded potted plants or cut flowers on an international scale making it a world's US$2 billion industry (Global Agriculture Information Network, 2010). Among the most popular orchid groups produced for the mass market are the *Dendrobium, Cymbidium, Phaphiopedillum, Cattleya, Phalaenopsis, Vanda, Arachnis, Ascocenda, Aranda and Oncidium.*

Taiwan is currently the world's number one orchid exporter, taking over Thailand since 2005 (Taiwan Floriculture Exports Association, 2010). The Taiwanese Government spent US$65 million to cover construction costs for orchid-related facilities and establishing the Taiwan Orchid Plantation that will create 1,500 jobs for its people (New York Times, 2004). Although the planting covers 4% of total floriculture are, the orchid sales covers 23% of the floral industry. In the first 10 months of 2010, Taiwan exported NT$2.39 billion (USD 77.66 million) worth of orchids. This is a 43% increase since 2009. From 2008-2010, sales of *Phalaenopsis* reached 8 million strains. *Oncidium,* the main species exported by Taiwan earned the nation US$7 million in 2006.

As an effort to dominate the *Oncidium* market, the government dedicated 190 hectares of land around the nation for the cultivation of this species (Taipei Times, 2006).As of the year 2012, the orchid export has increased 15-20% over a period of one year (China Times, 2012).

Thailand is the world's second exporter and produces 2,240 ha of orchids in production. For the past 40 years, Thailand has been developing its orchid breeding incessantly and rapidly. In 2001, Thailand exported US$50 million in cut orchids (Laws, 2002) and in 2004 continued to earn Thailand the highest income of all exported flowers earning US$60 million (Thai Farmer Research, 2004). In 2006, the value of Thailand's orchid export rose to a staggering US$80 million. The orchid export accounts for 80% of the total ornamental plants export of Thailand (Royal Flora Expo, 2009). In 2009 orchids topped the cut flower export industry with export worth more than USD80 million. This included 24,000 metric tonnes of cut flowers and 300,000 units of live orchid plants. In 2010, orchids made up 77% of all exported Thai floriculture products (Bangkok Post, 2011).

Singapore is also an important exporter, exporting over US$10 million worth of orchid flowers and plants to over 33 destinations including Japan, Australia, Greece, the United States and the Netherlands. Singapore supplies approximately 15% of the world market share for cut orchids, with an export range of around 60 varieties (Ministry of National Development, 2009).

Malaysia is a relatively new player in the world cut flower industry. Nevertheless, the market is growing steadily and its economic importance have been recognized by the Malaysian government.In the National Agricultural Policy (1992-2010), cut flowers, including orchids have been identified as a priority group of crops with the potential to meet domestic and international demands. Orchid production in Malaysia covers 300,000 hectares involving 36 growers (Ministry of Agriculture, 2011). In 2000, Malaysia's total orchid export was valued at RM33 million and rose to RM40 million in 2005. In 2009, orchids were worth 290 million ringgit in the floriculture industry in both the domestic and export market. The main importers of Malaysian orchids are Japan, Europe and the United States (Gus Wicjman, 2006).

### 2.1.2 Limited Shelf Life in Cut Orchids

Generally, orchids have a long life span, reaching a period of 4 months in some species and hybrids. However, certain conditions reduce the shelf life of orchids, cutting short a life cycle of several months down to a few days. For the cut flower industry, the major chemical pollutant affecting shelf life is ethylene (Halevy and Mayak, 1979). The most striking ethylene producing phenomenon in orchids is pollination. The fact that pollination is accompanied by production of ethylene and hastens senescence in orchids is a long accepted dogma. Pollination in orchids results in a cascade of dramatic visual, structural and cellular changes (Avadhani *et al.*, 1994), which ranges from colour change, wilting, hyponasty to changes in protein content and membrane destruction. It was observed that *Phalaenopsis* senesced within one day after pollination (Halevy, 1986).

van Doorn (1997) reported that various orchids exhibited symptoms such as wilting, permanent closure of perianth and colour change after pollination. In species where senescence occurs as a result of pollination, high sensitivity towards exogenous ethylene also occurs. *Phalaenopsis* sp. exposed to exogenous ethylene resulted in perianth closure while *Cattleya* sp. and *Cymbidium* sp. exposed to ethylene showed a clear change in petal colour (van Doorn, 2002).

Changes in sink-source relationships occur when cut flowers are detached from the main plant. The inexistence of the main plant as a source of energy forces cut flowers to function as a source rather than a sink organ (van Doorn, 2004). In order to fulfil this function, the recycling of nutrients and energy reserves occurs by breaking down and degrading lipids and protein as an alternative energy (Zhou *et al.,* 2005). Many parameters related to sink-source relationship, such as a decline of fresh weight in petals, dry weight, soluble (vacuolar) acid invertase activity and soluble carbohydrate content, are often linked to the onset of flower senescence in cut flowers.

Vase life depends to a great extent on the maintenance of a proper water balance in the flower. Water balance is a function of water uptake, water loss and water retention (Halevy and Mayak, 1979). Cut flowers are usually subjected to water loss either through transpiration or plugging of the vascular system by microbial growth in vase water. This phenomenon is very common in most cut flowers.

Besides bacterial growth, air emboli, and the deposition of substances in the xylem such as gums and mucilage are factors that also hinders water uptake by the stem (van Doorn *et al.,* 1991, and van Leperen *et al.,* 2000). A decrease in water uptake leads to wilting and water-stress that initiates a premature increase in ethylene synthesis, resulting in accelerated senescence (Coker *et al*., 1985).

## 2.2 BOTANICAL DESCRIPTION

The orchids belong to a group of flowering plants under the family *Orchidacae,* which includes several genera such as *Dendrobium, Phalaenopsis, Cymbidium, Vanda and Oncidium*. It is one of the largest groups among the plant families, and the highest evolved with the most complicated flower structure among the monocotyledons. (Mukherjee, 1983,). There are an estimated 20,000-25,000 species, which occupy wide ranges of ecological habitats and exhibit highly specialised morphological, structural and physiological characteristics (Dressler, 1990). Most of the orchids are perennial herbs with simple leaves, growing on tress as epiphytes or forest floor and open ground as terrestrials.

The orchid flower is zygomorphic in structure with seven floral parts -three sepals, three petals and the column. The unique structure of the orchid flower is its modified petal, known as the labellum or the lip. The lip is the most showy part of the flower and may be tubular as in the *Cattleya,* expanded with warts and protuberance as in *Oncidium*, or may be indistinguishable from other petals as in *Masdevallia.* The attractive nature of this structure acts as an attractor to pollinators (Arditti, 1979).

The column is the reproductive organ of the orchid and consists of the male (stamen) and female (stigma) organs. The stamen and the stigma are separated by a thin layer called the rostellum. The orchid flower exists in variations of shapes, colours and sizes earning orchids the reputation as one of the most beautiful and unique plant in the world.

The scientific classification of orchids is as stated below:

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| Kingdom | : | Plantae |
| Division | : | Magnoliophyta |
| Class | : | Liliopsida |
| Order | : | Asparagales |
| Family | : | **Orchidaceae** |

The *D.* Pompadour is an old cross of the hybrid *D.* Louis Bleriot (*D. phalaenopsis x D. superbiens*) with *D. Phalaenopsis* var schroederianum. The petals and sepals are rich crimson purple with a very dark patch in the center of the base of the middle lobe petal. The flower was first introduced in Thailand in 1934 and was the landmark that brought popularity to the orchids. The following figures show *D.* Pompadour orchids that are used in this study.

**Figure 2.1: *Dendrobium* Pompadour orchid flowers used in this study.** Flowers are shown as a potted plant (above left), inflorescence (above right) and a single flower (below).

## 2.3 FLOWER OPENING

The time of flower opening indicates the start of a flower's function to attract pollinators, leading to pollination and fertilization. The lifespan of these flowers are often terminated either by petal movement, petal withering and abscission.

### 2.3.1 Categories of Flower Opening

van Doorn and van Meeteran (2003) distinguished flower opening into two categories. Firstly, flower opening that is due to the development of tissue adjacent to the flower. Examples of this category of flower opening include the required growth of the pedicel and the forced separation of bracts and sepals in species of *Iridicae.* Flower opening may also be a result of the breakage of sepal margins that hold growing petals together during budding, eventually releasing the petals free (Sigmond, 1929). Secondly, flower opening that is dependent on petal movement. This movement is caused by either reversible ion accumulation due to water loss during the day and refilling at night or due to differential growth (van Doorn and van Meeteran 2003). Flower opening may be due to cell expansion as was demonstrated in *Gentiana kochiana.* The petals of these flowers contained an expansion zone that measured at 1-2 cm long and was capable of expanding at 1-3 cm beneath the petal tip. In *Ipomea,* flower opening is caused by the differential expansion of both sides of the petal midrib (Kaihara and Takimoto, 1981).

### 2.3.2 Carbohydrate Metabolism during Flower Opening

In most species, flower opening is accompanied by mobilization of carbohydrate and translocation of sucrose. In the young petals of *Alstromeia, Lilium, Rosa*and *Magnolia,* starch is rapidly converted into glucose and fructose shortly before flower opening (Ho and Nichols, 1977). Starch hydrolysis not only provides substrate for respiration to occur, but is also crucial to provide osmotic solutes for water influx and cellular

expansion. (van Der Mueler-Muisers *et al.,* 2001). On the other hand, in some species, conversion of starch did not seem to be the source of sugars prior to flower opening. In unopened daylily, fructan was present in high concentration while starch was not present and it was rapidly degraded upon flower opening. The degradation of both starch and fructan has been reported during flower expansion in chrysanthemum (Trusty and Miller, 1991). In flowers such as *Sandersonia* (Eason *et al.,* 1997) the increase of glucose and fructose is due to sucrose uptake from the apoplast as neither or very low fructan or starch concentration was found.

### 2.3.3 Ultra Structural Changes during Flower Opening

Flower opening is accompanied by a high rate of cell wall expansion. Turgor changes occurring in epidermal cell have been suggested to be the main cause of expansion. However, considering rigid cell wall blocks water influx into the cell, there is a need to loosen cell walls in order for expansion to occur. In tomato flowers, high expression of expansins were observed, which are crucial for the loosening of cell walls (Brummel *et al.,* 1999).

In roses, xyloglucan hydrolase, a protein also responsible for protein cell loosening was found to be expressed prior to flower opening (Takahashi *et al.,* 2010). In *Ipomea* flowers, cell wall enlargement and wall expansion are accompanied by substantial reduction in outer cell wall thickness and increase in the size of the central vacuole (Phillips and Kende, 1980). Several interesting features of cell expansion were observed in Asiatic lily through microscopy, which includes the development of internal air spaces during bud growth, tissue breakdown before full flower opening, constant expansion of the inner and outer epidermal cells at different times and the decrease in

midrib width around flowering time that may be the overlying factor of sepal release (Bieleski *et al.*, 2000).

### 2.3.4 Pigment Development during Flower Opening

The development of colour during flowering ensures that the flowers remain attractive to pollinators so as to ensure successful pollination. Flower colour is determined by two major factors, pigments present in the vacuole, and the intravacuolar environment. In *Sandersonia aurantica* flowers, the main change observed during the development from bud to anthesis were loss of chlorophyll, B-carotene and lutein, and the increase in zeaxanthin and crytoxanthin, which gives the opened flower its orange colour. In petunia flowers, the change in colour during bud development to floral anthesis was accompanied by a decreasing trend of vacuolar pH, and was reflected in the increased activity of the tonoplast $H^+$ ATPase (Grotewall, 2006).

## 2.4 SENESCENCE

### 2.4.1 Introduction to Plant Senescence

Senescence is a phenomenon that has always allured major debates among scientists in the search for an accurate definition and the biological processes that it entails. Among the earliest attempts to define senescence was by Medawar (1952) who defined senescence in animal biology as having death as its end point. This definition was later extended to plant biology when Leopold (1961) defined senescence in plant cells as "the deteriorative processes that are the natural causes of death". Sacher (1973) then defined senescence as "the final phase in the ontogeny of the organ in which a series of normally irreversible events are initiated that lead to cellular breakdown and death of the organ".

Although there is a general acceptance towards the definition of senescence, nevertheless confusions arise in distinguishing between ageing and senescence, since ageing in plants is a process that also ends in death. Leopold (1980) referred to ageing as the gradual deterioration of an organism with time and age, thus depending on the strength or the weakness of an organism to survive. Senescence on the other hand, is a highly regulated event where changes occur due to specific signals to ultimately result in death (Leopold, 1980).

One of the famous definitions of PCD originated from Zhivotovski *et al.,* (1997) who defined PCD as "a genetically controlled cell deletion process". Laytragoon (1998) emphasized that PCD is a process whereby developmental or environmental stimuli activate a "specific series of event that culminate in cell death". Opinions exist that the interchangeable usage of the term senescence and programmed cell death is rather inaccurate. Delorme *et al.,* (2000) and Thomas *et al.,* (2003) proposed that senescence is a process that occurred before plants reached death. An example of this would be the process of leaf yellowing (also referred by some as senescence), where reversibility exists and regreening can occur. Redefining senescence as a trajectory to death led to a number of oppositions. Firstly, it was already accepted by most plant biologists that senescence would lead to death and is widely used when describing root , petal and fruit senescence. Secondly, it was argued that reversibility of senescence is rare in plants, and even if it existed, would only be found in leaves and shoots. The acceptance of senescence as a process that need not necessarily result in eminent death would therefore mean that other organs such as petals, roots, stigma and anther only experience PCD, and not senescence.

Nooden *et al.,* (2004) on the other hand, suggested that while senescence refers to the death of organs or a whole plant, PCD refers to the death of a relatively small number of cells. This reference also has its fair share of objections. van Doorn and Woltering (2004) argued that there is no necessity to distinguish between cell death in a whole plant and death by a small group of cell. Senescence can be applied to refer to death of cells in a particular organ and whole individuals.

Moreover, the morphological changes which are visible during senescence are the results of the cell death programme. All these arguments point to the fact that senescence and PCD are very similar processes with the same end point; death and could therefore be accepted as synonyms.

### 2.4.2 Petal Senescence as a Programmed Cell Death

Flowers are the structures for sexual reproductivity which is vital for the continuity and propagation of plants. The lifespan of flowers on plants is limited and is species-specific to adapt to its environment. This limited lifespan is required as the flower is a substantial sink-organ that demands costly maintenance in terms of energy usage (Ashman and Schoen, 1994). Furthermore, the stigma of flowers is a point of entry for pathogens, thus making the flower an added risk to the whole plant (Shykoff *et al.*, 1996). Considering the flower is a liability to the plant, there is no need for the flower to be maintained beyond its usefulness. Petal senescence occurs to ensure that the flower is discarded when it is deemed to have fulfilled its function. The importance for the plant to strictly regulate the death of its flowers makes it obvious that a tightly controlled programme for petal senescence must exist. It can be triggered by several factors including stress, wounding and most common, pollination. Events that accompany petal senescence have been reported in numerous papers, showing a genetically based programme characteristic to programmed cell death (Zhou *et al.,* 2005).

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### 2.4.2.1 Molecular Regulation of Senescence

Senescence is a deteriorative process that involves remobilization and translocation of resources within the organ or whole plants in order to achieve death. Conventional theories states that these processes are random, uncontrolled biochemical processes within the plants, resulting in cell lyses by hydrolytic enzymes. However, over the years, evidence of a controlled death process at the level of transcription and translation has been established.

On a molecular level, the regulation of senescence is accompanied or driven by changes in gene expression. One of the earliest demonstrations of gene expression during senescence was carried out by Watanabe and Imaseki (1982) on the subject of leaf senescence. They elucidated that an abundance of leaf mRNAs were significantly down-regulated during senescence. In carnations, expression of several mRNAs was found to be up-regulated during the course of petal senescence (Woodson *et al.*, 1992).

As senescence is a deteriorative process, decline in protein, RNA synthesis and lipid destruction is an expected hypothesis. This hypothesis is supported by findings that reveal the up-regulation of protease (Jones *et al.,* 2005; Wagstaff *et al.,* 2002) and lipase (Hong *et al.,* 2000), which is simultaneous with senescence in a variety of plants. Up to date, more than 30 senescence associated -genes have been identified in a variety of species and the functions demonstrated range from encoding degradative enzymes to genes that control the translocation of nutrients.

*SAG12*, a gene encoding a cysteine protease discovered in *Arabidopsis*, is specifically activated by developmentally controlled senescence pathways but not by stress- or

hormone controlled pathways (Noh and Amasino, 1999). Six genes were found to be highly up regulated during senescence in daylilies and were identified as SAGs, known as DSA1 to DSA6 (Panavas *et al.,* 1999). In *Alstroemeria,* a putative cysteine protease showed a dramatic increase during petal senescence and may be responsible for encoding an important enzyme for the proteolytic process in this species (Wagstaff *et al.,* 2001). Grbic and Bleecker (1995) reported that ethylene is a modulator in leaf senescence hence mutants of *Arabidopsis* showed longer leaf longevity. They elucidated that the blocking of ethylene perception in this plant probably lead to the delay in the production of senescence associated genes (SAGs).

**2.4.2.2 Hormonal Regulation of Senescence**

Abscicic acid (ABA) has been implicated in leaf senescence and exogenous application of this hormone has been shown to hasten senescence in rose (Borochov and Woodson, 1989) and carnations (Vardi and Mayak, 1989). However, ABA does not necessarily hasten senescence in all plants. As demonstrated in roses, no increase of ABA was observed during senescence. Instead, a reduction of ABA was exhibited after the roses were cut. Increase of ABA was only observed after the termination of vase life.

Besides ABA, ethylene has also been established as a hormone that promotes senescence in plants. Plants where ethylene promotes senescence, known as ethylene sensitive plants undergo a climacteric phase where a burst of ethylene production supersedes senescence and exposure to exogenous ethylene hastens senescence. In 1974, Kende and Baumgartner reported that curling of petals, a manifestation of senescence in *Ipomoea tricolor* could be induced prematurely by treatment with ethylene. Work done by Suttle and Kende (1978) showed that production of endogenous ethylene sharply increases in *Tradescantia* flowers as they deteriorate and exogenous

ethylene accelerates the rate of senescence in these flowers. In carnation flowers, senescence is also accompanied by increase in ethylene. In senescing carnations, ethylene is initially produced in the ovary and subsequently translocated to other flower parts (ten Have and Woltering, 1997), a phenomenon also observed in senescing *Cymbidium* (Woltering *et al.,* 1993) where ethylene was implicated as the mobile factor responsible for petal senescence.

On the contrary, ethylene insensitive plants do not produce ethylene prior to senescence and exposure to exogenous ethylene does not affect changes in plant longevity. Lilies, for example are characterized ethylene- insensitive, as they do not appear to produce endogenous ethylene, or respond to exogenous ethylene. In *Alstroemeria* flowers, senescence occurs independent of endogenous ethylene production. However, it is interesting to note that the completion of flower abscission in this flower requires a small burst of ethylene (Wagstaff *et al.,* 2005).

For ethylene-insensitive flowers, ABA and cytokinins are possibly the main signals for triggering senescence in petals. Treatment with ABA hastens senescence associated events, such as ion leakage and lipid peroxidation (Panavas *et al*., 1998). The hormones cytokinin and gibberellins that are naturally produced in plants regulates inhibition and delay of senescence. Cytokinin functions in the maintenance of chlorophyll, RNA and proteins that decline during senescence. Gibberellins act as suppressors of chlorophyll loss in leaves, fruits and stalks and the production of this hormone coincides with cytokinin.

### 2.4.2.3 Cell Wall and Membrane Destruction

Alterations on cell wall constituents during senescence result in changes in cell shape and size. Senescence of *Sandersonia* flowers was accompanied by changes in cell wall polymers such as cellulose and pectins (Vetten and Huber 1990; O'Donoghue *et al.,* 2002). As a result, structural integrity of flowers is lost. Alterations in these cell wall components may serve several purpose: i) changing degree of cell-to cell attachment ii) remobilization of materials to persisting organs (Panavas *et al.,* 1998). These observations are similar to that reported in ripening fruits where softening occurs as a result of cell wall destruction (Chin *et al.,* 1999; Deng *et al.,* 2005). The various cell wall modifications are caused by the actions of a range of cell wall modifying enzymes, which include but are not limited to polygalacturonase (PG), pectatelyase (PL), pectin methylesterase (PME) and cellulose. Activity of cell wall hydrolases however, occur at varying degrees in different flowers. For example, increasing PG activity accompanied rapid pectin hydrolysis in daylily (Panavas *et al.,* 1998) while PG and PME activity was low in *Sandersonia* flowers (O'Donoghue *et al.,* 2002). The application of synthetic cell wall hydrolases, pectinase and PG to the basal ends of daylily flowers was found to stimulate petal senescence. Lipid peroxidation and membrane digestion were evident in the flowers treated with synthetic hydrolases (Panavas *et al.,* 1998).

Cell membranes function as a gateway that allows the cell to regulate the transportation of nutrient into the cell. The structure and internal compartmentalization of the cell is also maintained by the integrity of the membrane. During petal senescence, one of the most obvious changes that occur is the change in the membrane integrity and structure (Matile and Winkenbach, 1971). The profound changes in cell shape and size indicates constituents of the cell wall are altered during senescence. Morning glory columnar cells become rounded and the cell wall is reduced in thickness and protrudes to the

nearby lumen. Invagination of tonoplast has been reported in senescing morning glory petals. Furthermore, increasing volume of cytoplasm at the expense of the vacuole was also observed, indicating a loss in differential permeability (Phillips and Kende, 1980). In the later stages of senescing carnation (post-climacteric), the membrane ruptures and cytoplasmic debris is evident in the intracellular spaces (Smith *et al.,* 1992). Accompanying the changes in membrane structure are the changes in biochemical properties within the cell. A simultaneous decline in all classes of phospholipids is characteristics of petal senescence. This is a result of greater phospholipase and acyl hydrolases activities enzyme (Paliyath and Droilard, 1992). Furthermore, data obtained from senescing daylilies also suggest that the production of phospholipids during petal senescence is also blocked (Beiliski and Reid, 1992). At the molecular level, an up-regulation of genes responsible for the production of hydrolases and the down-regulation of genesresponsible for phospholipids synthesis maybe direct cause of membrane destruction (Wang *et al.,* 1993).

#### 2.4.2.4 Carbohydrate Changes

Recycling of nutrients and energy reserves are characteristics of senescence as a form of programmed cell death. Flowers are switched from being a sink plant, receiving energy and nutrients from the mother plant, to a source plant where energy and nutrients are remobilized and translocated within the flower. Many parameters linked to sink-source relationships such as decline in fresh weight in petals, dry weight, and soluble carbohydrate are observed in senescing flowers. Senescing flowers exhibit a gradual decline in respiration which is attributed to a shortage in available respiratory substrates (van Doorn, 2004). The final stage of flower development is characterized by a decline in the content of carbohydrates and dry weight of petals (Coorts, 1973). Loss of dry

weight has been observed to coincide with reduced size of starch bodies as well as starch content (Lesham *et al.,* 1986).

In senescing cut carnations, daylilies and *Sandersonia* flowers, a considerable loss of total carbohydrate was observed from the petals (de Vetten and Huber, 1990; Beiliski, 1995; Wagstaff *et al.,* 2002). In cut carnation flowers, where the source of carbohydrate supply from the main plant is eliminated, petals translocate sugars to ovaries, resulting in an increase in the dry mass of ovary. According to van Doorn (2004), upon sugar starvation, expression of genes involving the breakdown of starch and protein is enhanced while the genes involved in the synthesis of amino acids and amides are actively transcribed. It has been suggested that levels of carbohydrate, especially sucrose is a determining factor in the longevity of flowers. The difference in sink strength may be the reason why some flowers live longer than others.

#### 2.4.2.4 Protein Changes

Protein degradation has been reported to be a common feature of senescence (Hensel *et al*., 1993; Callis, 1995; Beers and Freeman, 1997). Decrease in total protein levels has been found in both ethylene sensitive and insensitive flowers. A culmination of decreased synthesis and increased degradation results in an overall loss of proteins during senescence (Celikel and van Doorn, 1995). It was suggested that protein is degraded by pre-existing cytosolic, vacuole or cell wall proteinase and at the same time new proteinases are synthesized (Callis, 1995). Inhibition of *de novo* protein synthesis using CHI results in application for delay the flower senescence of many flowers such as carnation (Wulster *et al.,* 1982), daylily (Lay-Yee *et al.,* 1992), and Narcissus (Jones *et al.,* 1994).

Furthermore, the application of CHI was also found to improve flower appearance, membrane integrity, electrolyte content and supress ethylene production (Drory *et al.*, 1995). Products of protein degradation are remobilized from senescing organs to other parts of the plants (Rubinstein, 2000). In *P. amabilis*, *D. nobile and C. labiate*, protein remains balanced until death. However, accelerated senescence induced by pollination results in rapid degradation and subsequent translocation of nitrogen from the senescing perianth to the developing ovary (Hsiang, 1951; Arditti, 1979). It was suggested that hydrolysis occurs in senescing organs as opposed to protein synthesis in persisting organs. The onset of excessive degradation of proteins was reported to coincide with the depletion of sugars in senescing cut *Lupinus* (Weinstein and Laurencot, 1958). van Doorn (1995) suggested that breakdown of proteins during flower senescence may also serve as an alternative source of energy when supply of respirable substrates in the form of sugar is depleted.

### 2.4.3 Pollination- induced Petal Senescence (PIPS)

A flower's attraction or longevity is nature's way of attracting pollinators to ensure successful pollination and propagation of species. Arditti (1979) explained that because orchids are pollinated by a specific vector, the number of pollinators is limited. Attractive flowers and longevity are therefore adaptations that would provide a longer "waiting period" to ensure successful pollination. Orchid flowers may live up to several months as is the case with *Paphiopedilum* flowers which live up to nine months (Arditti, 1979).

Once pollinated, flowers undergo numerous changes in perianth structure, such as wilting or abscission, perianth colour change or cessation in nectar production (Stead, 1992; Porat *et al.*, 1994). All these changes render the flowers unattractive and eventually eliminate the functional life of flowers.

More often than not, pollinated flowers exhibit hastened wilting or senescence and reduced longevity compared to that of unpollinated flowers. A number of advantages of the reduction in longevity were suggested by Stead (1992):

1) To avoid further deposition of pollen which could be wasteful for potential breeding
2) To eliminate excessive pollen tube growths which could result in nutrient competition
3) To minimize respiratory and water transpiration, two costly processes involved in the maintenance of floral structure

Pollination-induced senescence (PIPS) in orchids may be first described in 1793 by Conrad Sprengel (Arditti, 1979). Since then, it has continued to receive great attention and many literature have focused on different aspects of PIPS in *Orchidaceae* and other flowers ranging from the relationship of pollinators and flowers to the post-pollination events that follows (Hurst, 1898; Dodson,1967; Knauft *et al.*, 1970; Nadeau and O'Neill, 1993; van Doorn, 1997; Yu and Goh, 2001; Chandran *et al.*, 2007*;* for review see Avadhani *et al.*, 1994).

In most flowers, senescence is characterized by the in rolling of petals, and eventually results in permanent flower closure. Permanent flower closure have been documented as a distinct characteristic of PIPS in different species of orchids such as in *Arachnis* (Hew *et al.*,1989), *Phalaenopsis*, (Porat *et al.,* 1994) and *Dendrobiums* (Ketsa and Luangsuwalai, 1996).

It has been a long accepted dogma that PIPS is induced or accompanied by enhancement of ethylene production by the flower, and that ethylene can accelerate flower senescence (Stead, 1985). In many species, an increase in the production of ethylene is the earliest detectable biochemical events in the pollinated stigma, often occurring a few hours after pollination. In *Cymbidium* Jungfrau orchids, evolution of ethylene was first detected in the rostella 5-6 hours after application NAA (Chadwick *et al.,* 1986). An interorgan translocation of ACC from the stigma to other floral segments has also been reported in *Phalaenopsis* (Lizada and Rimando, 1985) and *Cymbidium* Jacobi (Woltering, 1990). Jones and Woodson (1997) reported that there are three distinct peaks of ethylene production in styles of carnations: first a small peak one hour after pollination, then two more peaks, at 36 and 48 hours after pollination. This increase is then transmitted to the rest of the flower parts and is postulated to serve as a coordinator for the subsequent post pollination events.

Initial investigations led to the postulation that the symptoms exhibited by PIPS are related to the desiccation- induced ethylene production following wounding of the stigma or the rostellum. This observation was supported by the fact that emasculated flowers where the anther was removed also resulted in similar ethylene production, although the amount of ethylene production might not necessarily be the same (Halevy *et al.,* 1984). Furthermore, treatments with ethylene biosynthesis inhibitors such as

aminooxyacetic acid (AOA) and aminoethoxyvinyl glycine (AVG) have shown to prevent accelerated senescence in pollinated orchids (Nair and Tung, 1980; O'Neill *et al.,* 1983; Chandran *et al.,* 2005, 2007), carnations (Larsen *et al.,* 1995) and petunias (Hoekstra and Weges, 1986).

Although the production of ethylene in pollinated flowers was found to be consistent in many ethylene sensitive flowers, the levels of ethylene production vary. This led to the need to identify the chemical stimulant that was responsible in endogenous ethylene production in pollinated flowers. Whitehead *et al.,* (1983) demonstrated that this biochemical stimulus was in the form of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) found in the pollen. According to Ketsa (1996), a positive correlation exists between the concentration of ACC in pollinia and the rate of petal senescence. His observation on different cultivars of the *Dendrobium* orchids supported the findings of Halevy (1986) where flowers pollinated by pollinia containing a greater concentration of ACC content where found to induce a higher rate of ethylene production, which in turn resulted in an earlier senescence of flowers. As is demonstrated by ethylene, ACC too have been found to originate from the receptive stigma and then translocated to other parts of the flowers. High concentrations of ACC content in the column have been detected compared to that of the petal or sepal, suggesting that the bulk of ethylene production is in the stigma (Pech *et al.,* 1987).

In an attempt to understand the molecular regulation of pollination-induced ethylene production, a number of studies have focused on the enzymes that convert the ethylene precursor into ethylene. O'Neill *et al.,* (1993) reported that pollination of *Phalaenopsis* flowers promoted ACC synthase and ACC oxidase mRNA accumulation. The accumulation of the two enzyme mRNAs however was not observed in the petals

although the petals make up the biggest part of the flower. This finding supports the model of an inter-organ regulation ofpost pollination development that depends on pollination-stimulated accumulation of mRNA encoding ethylene biosynthetic enzymes in a developmentally regulated and tissue-specific manner. Grierson *et al.*, (2000) concluded that the expression of ACC oxidase and ACC synthase genes and the translocation of ACC originating from the pistils are important for regulating the initial burst of ethylene production in pollinated tomato flowers.

## 2.5 ETHYLENE

### 2.5.1 Ethylene Biosynthesis

#### 2.5.1.1 Ethylene Biosynthesis Pathway

The study of ethylene pathway has been elucidated in many studies, most prominently by Yang and co-workers (Adams and Yang, 1979). The first step in the pathway is the conversion of methionine and ATP to S-adenosylmethionine (SAM), catalysed by the enzyme SAM synthetase. SAM is converted by ACC synthase to 1-aminocyclopropane-1-carboxylic acid (ACC) and 5'-methylthioadenosine (MTA). MTA can be recycled to methionine, thus allowing high rates of ethylene production even if methionine concentrations are low. ACC is the immediate precursor of ethylene. Oxidation of ACC by the enzyme ACC oxidase (previously known as EFE, the ethylene-forming-enzyme) results in the production of ethylene, $CO_2$ and HCN.In the course of the cycle, one molecule of ATP is expended, and the breakdown product of ACC can be used to synthesize a new methionine molecule (Miyazaki and Yang, 1987).

Ethylene production is retarded when plant tissues are placed under anaerobic conditions and results in a definite increase in ethylene production when re-exposed to air. This is an indication that there exists an accumulation of an intermediate during the anaerobic phase, which subsequently is converted to ethylene when exposed to air. This compound was later identified as 1-aminocyclopropane-1-carboxylic acid (ACC) that has now been universally accepted as the immediate precursor to ethylene (Kende, 1993). Other than its conversion to ethylene, ACC can also be metabolized to produce a non-volatile compound known as N-malonyl-ACC (MACC). Although this conversion is reversible and the conjugate does not easily form ethylene, MACC is thought to be an inactive end product rather than a storage form of ACC representing an important mechanism for the regulation of ethylene production (Beyer *et al*., 1984).

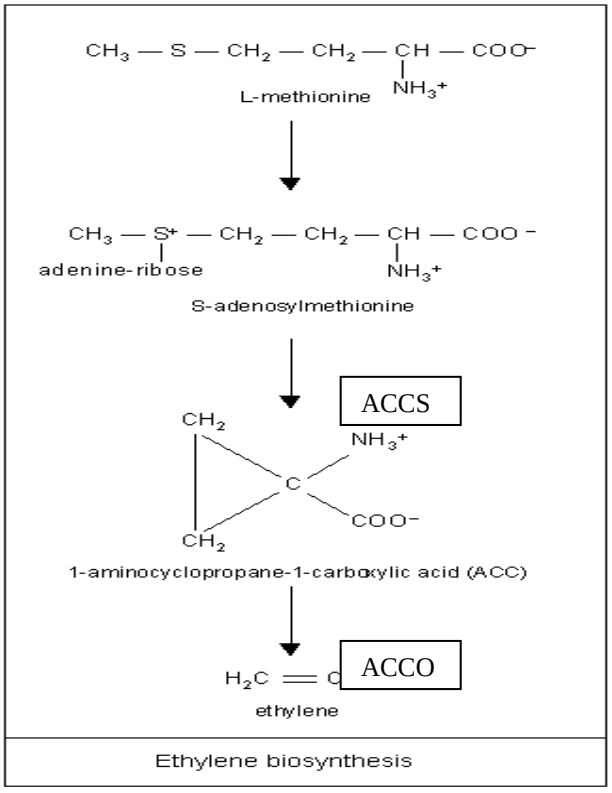


**Figure 2.2 Ethylene biosynthesis pathway as elucidated by Yang and Hoffman, (1984).**

#### 2.5.1.2 Ethylene Biosynthesis Enzyme Genes

The two enzymes central in the ethylene biosynthesis pathway are ACC synthase (ACCS), which catalyzes the conversion of S-adenosyl-methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid, and ACC oxidase (ACCO) which catalyzes the conversion of ACC to ethylene. ACCS belongs to the family of pyridoxal 5'-phosphate-dependant enzyme. This enzyme is part of a multigene family and transcription of different forms is induced by different physiological and environmental conditions (Stearns and Glick, 2003). ACCS is generally accepted as the rate-limiting step in the ethylene biosynthesis pathway (Yang and Hoffman, 1984) and was first identified in ripening tomatoes in 1979. The first *ACCS* cDNa ever cloned was from zucchini fruit tissues and over expressed in *E. coli* and yeast (Zarembinski and Theologis, 1994). In *Arabidopsis thaliana*, genomic sequences allowed the isolation and characterization of five *ACCS* genes. The genes were differentially expressed and responded to environmental stimuli (Theologis *et al.,* 1992). In a study done by Bui and O'Neill (1998) on pollinated *Phalaenopsis* orchid flowers, the expression patterns of three synthase genes were found to be time and space dependant. The study established the existence of an inter-organ regulation of these genes which originates from the stigma where the pollination signal is initiated and subsequently translocated to the perianth.

Compared to *ACCS*, little is known about the role of *ACCO* in regulating the biosynthesis of ethylene. *ACCO* genes are part of a multigene family, and exist in a number of isoforms. These genes, which are responsible for the conversion of ACC to ethylene are present in most tissues at very low levels. *ACCO* was first isolated and cloned from tomatoes and was name pTOM13 (Holdsworth *et al.,* 1987). Anti-sensing this gene resulted in reduced levels of ethylene production in tomatoes, and was then identified as the gene which encodes for the *ACCO* enzyme (Hamilton *et al.,* 1990).

Studies on the regulation of *ACCO* expression in pollinated-induced senescence of a hybrid *Phalaenopsis* orchid, has shown that the increase in ethylene production corresponds to *de novo* synthesis of the protein after pollination. Furthermore, transgenic carnations containing antisense *ACCO* exhibited a significant delay in case life, concomitant with a reduction in ethylene production (Savin *et al.,* 1995).

#### 2.5.1.3 Spatial and Temporal Regulation of *ACCO* and *ACCS* Genes

An inter-organ translocation of ethylene within the floral organ has been reported in a number of flowers (O'Neill *et al.,* 1993; Woltering, 1990; ten Have and Woltering, 1993). Arditti (1979) reported that ACCO activity levels rise initially in the column, where pollination is first perceived, and is subsequently translocated to the petals and sepals. Grierson *et al.,* (2000) later concluded that the expression of *ACCO* and *ACCS* genes and the translocation of ACC originating from the pistils are important for regulating the initial burst of ethylene production in pollinated tomato flowers.

It is interesting to note that three *ACCS* genes, *Phal-ACCS1*, *Phal-ACCS2*, and *Phal-ACCS3,* are differentially expressed in the pollinated orchid flower as reported by Bui and O' Neill (1998). In carnations, similar observations were made where three members of the *ACCS* genes (*DC-ACCS1, DC-ACCS2* and *DC-ACCS3*) were found to be differentially expressed during pollination (Jones and Woodson, 1999). In the stigma of carnations and petunias, the *ACCO* genes were found to be present in a constitutive state, indicating that pollination- induced ethylene in the stigma appears to be largely controlled by *ACCS*, rather than *ACCO*. However, a rather intriguing observation was obtained in the study done by Nadeau *et al.,* (1993), which reported that *ACCO* gene expression levels found in the stigma increased after pollination. The question that begs

to be answered is whether the non-constitutive state of *ACCO* reported in *Phalaenopsis* is present in *D.* Pompadour.

### 2.5.2 Ethylene Signalling

#### 2.5.2.1 Ethylene Receptor Genes

Petal senescence is a process regulated by a complex of ethylene biosynthesis and perception. The insight into ethylene perception was realized by studies using *Arabidopsis thaliana* as the model plant. Plants grown in the dark in the presence of ethylene exhibited three distinct characteristics, known as the "triple response": exaggerated apical hook formation, inhibition of root and hypocotyl elongation, and swelling of the hypocotyl (Guzman and Ecker, 1990).

These morphological characteristics allowed for screening and the categorization of plants based on their sensitivity to ethylene. This has led to the cloning of corresponding genes and the elucidation of an ethylene perception and transduction pathway involving a number of receptors. To date, five members of the ethylene receptor family have been identified in *Arabidopsis thaliana; ETR1, ERS1, ETR2, ERS2* and *EIN4*. The deduced proteins of these 5 receptors share 57-79% sequence similarity to each other, with the amino terminal being the most conserved region (Hua and Meyerowitz, 1998).

The receptors are homologous to a two-component bacterial histidine kinases involved in responses towards environmental change. A histidine kinase domain acts as a sensor where autophosphorylation occurs, while a sensor domain regulates downstream responses (Kende, 1989). The ethylene receptors, on the other hand, are made up of three main domains, the sensor domain, the kinase domain and the receiver domain. The sensor domain consists of three hydrophobic, putative transmembrane stretches. *ETR2,*

*ERS2* and *EIN4* may have a fourth membrane spanning domain. Ethylene binding occurs within this domain. Initial ideas suggested that the binding of ethylene to these receptors was through a transition metal and was reversible (Burg and Burg, 1967). This idea was proven with the identification of *ETR1,* as a protein capable of reversibly binding to ethylene (Bleecker and Schaller, 1996). Ethylene binding also appears to be mediated through a copper cofactor delivered by *RAN1* (Hirayama *et al*., 1999; Rodriquez *et al*., 1999).

At the C terminal end of this domain, lies the GAF domain (small molecule binding units). As of now, the function of this domain in ethylene receptors remains unknown.
The kinase domain has very high sequence homology to His Kinase and consists of five subdomains. *ETR1* and *ERS 1* are categorized in the subfamily 1 receptors as both receptors have a well-conserved His-kinase domain. On the other hand, *ETR2, ERS2* and *EIN4* (subfamily 2) lack some of the His-kinase hallmarks. The question then arises as to whether His-kinase is needed for receptor signalling. Recently, it was shown that a transformation of an *ers1-2* mutant with histidine-inactive form of *ETR1* was able to restore normal growth in the double mutant seedlings. Thus, concluding that canonical His-kinase is not needed in ethylene signalling (Wang *et al.,* 2003). The lack of conservation of this domain could be explained by the discovery of Ser/Thr kinase (STK) activity found in the receptors of *Arabidopsis* (Moussatche and Klee, 2004).

The receiver domain share sequence identity with the output portion of bacterial two-component systems and contains an Asp that is active in phosphorelay in bacterial proteins. However, not all of the ethylene receptors contain this domain as *ERS1* and *ERS2* were found to be lacking the receiver domain (Muller and Stunman, 2003).

### 2.5.2.2 Ethylene Signal Transduction Pathway

Ethylene receptors function as negative regulators as the binding of ethylene results in the inactivation of receptor function. In the absence of ethylene, the receptors are found to be in an active state, interacting with *CTR1* (constitutive triple response factor*)* and repressing downstream ethylene response. Loss-of-function mutations in *CTR1* result in global constitutive activation of all ethylene responses examined (Kieber *et al*., 1993). Upon binding of ethylene, the receptors become inactivated and undergo conformational change in *CTR1*. This causes disassociation of receptors with *CTR1*, which in turn releases the suppression of the MAPK cascade and results in the activation of *EIN2*. *EIN2* is an integral membrane protein with 12 putative membrane-spanning domains and exhibits high similarity with a mammalian metal transporter. Subsequently, the activation of transcription factors, *EIN3* and *ERF1* initiates the ethylene response (Guo and Ecker, 2004).

Several studies have shown that the ability to perceive ethylene may be based on a collective level. Loss of function single mutants in the receptors found in *Arabidopsis thaliana* did not result in any defects in ethylene response, whereas when three or four of these genes were mutated, constitutive ethylene response phenotypes were observed. One argument that explains this rather puzzling phenomenon was suggested by Guo and Ecker (2004), arguing that triple and quadruple mutants exhibit constitutive ethylene response because when there are less receptors functioning, the basal level of constitutive ethylene produced in these plant are sufficient enough to inactivate the remaining receptors. This model assumes that there is a threshold effect of ethylene. Expression studies done on different rose cultivars, cultivar ‘Bronze’ and cultivar ‘Vanilla’ showed that the vase life of these flowers were negatively correlated with the expression patterns. It was observed that the bronze cultivar, which exhibited a shorter

vase life showed a distinctly higher expression of *RhETR1* compared to that of the 'Vanilla' cultivar, which had a longer vase life (Muller *et al.*, 2000).This observation however, is contradictory to the standard model of ethylene signal transduction, which predicts that lower receptor numbers increase sensitivity while increased receptor numbers would result in the opposite effect (Bleecker and Kende, 1999).

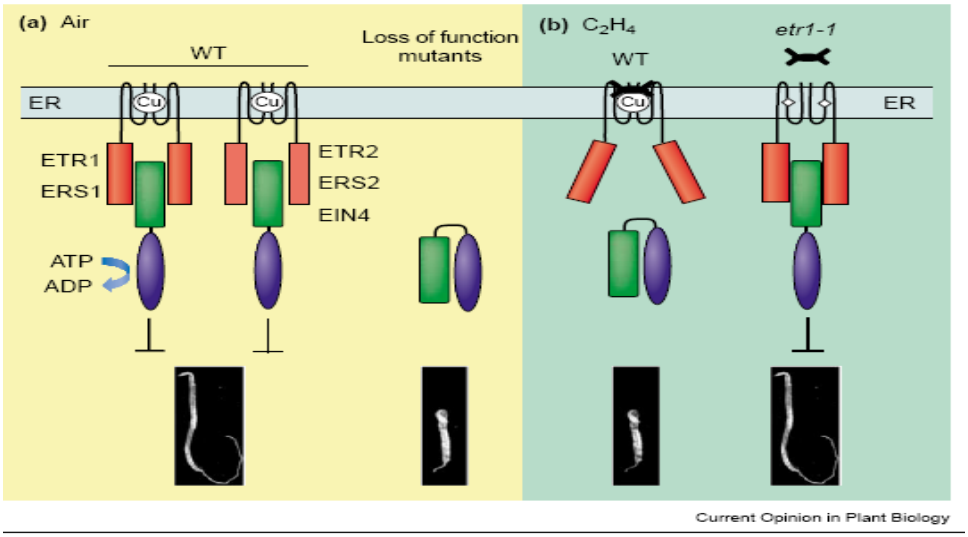


**Fig 2.3 A proposed model for ethylene perception (Guo and Ecker, 2004).** Without ethylene, the receptors remain activated and associated with *CTR1*, suppressing downstream ethylene response. The suppression of ethylene response is also conferred by *etr1-1 Arabidopsis* mutants. Upon binding of ethylene, ethylene receptors undergo structural change, releasing *CTR1*, and activating downstream ethylene response. This is conferred by loss of function *Arabidopsis* mutants.

### 2.5.2.3 Spatial and Temporal Regulation of Ethylene Receptor Genes

During the process of floral senescence, ethylene receptors are expressed differentially in different parts of the flower and at different times. Northern analysis on *ETR1* and *ERS2* mRNA in carnations revealed that *DC-ERS2* in petals decreased as the senescence progresses, increase slightly in ovaries and remained unchanged in styles throughout senescence. *DC-ERS1* and *DC-ETR1* increased inversely with the increase of ethylene production in the flowers. This inverse relationship may be the reason for increased ethylene sensitivity during senescence, thus accelerating senescence. *DC-ERS1*, however, was not detected throughout senescence (Shibuya *et al.*, 2002). In ethylene-

sensitive chrysanthemum “Iwa-no-hakusen” hybrid, levels of *ERS* increased in the petals as the onset of senescence began. The *ERS1* level exhibited a marked decrease 8 days after the onset of senescence (Narumi *et al.,* 2005). This observation supports the hypothesis of inverse ethylene-receptor relationship as senescence progresses. In geranium, where two ethylene receptors *PhETR1* and *PhETR2* have been isolated, it was found that transcript levels of these receptors remain unchanged within petals, sepals and pistils throughout floral development. The transcripts continue to remain constant and unaffected by self-pollination and ethylene treatment (Dervinis *et al.,* 2000).

This observation contradicts the observation reported in carnation and chrysanthemum where varying levels of receptors are present at the different parts of the floral organ. Instead, the results indicate that levels of ethylene receptors may not be an indication of floral sensitivity to ethylene. This could also mean that there may be another member of the *ETR* family that is present and mediates senescence in geranium either at the post-transcriptional level or via downstream component of the transduction pathway (Dervinis *et al.,* 2000).

## 2.6 EXTENDING CUT FLOWER SHELF LIFE WITH CHEMICALS AND PRESERVATIVES

### 2.6.1 Ethylene Inhibitors

The deteriorating effects of ethylene on post harvest characteristics can be prevented via two approaches:

1) by inhibiting the plant’s own production of ethylene
2) by blocking the binding of ethylene to receptors

Throughout the years, several chemicals have been developed and used to interfere with the biosynthesis of ethylene or inhibit ethylene action.

#### 2.6.1.1 Ethylene Biosynthesis Inhibitors

Ethylene biosynthesis inhibitors functions by interfering with the ethylene biosynthesis pathway.More often than not, these inhibitors share similar structures with methionine, the immediate precursor of ethylene synthesis, or by inhibiting the conversion of SAM to ACC.

##### 2.6.1.1.1 Aminooxyacetic acid (AOA)

Aminooxyacetic acid (AOA) is a very potent inhibitor which forms a complex with the cofactor pyridoxal phosphate, thus disallowing ACCS activity and the conversion of SAM to ACC (Keller and Volkenburgh, 1997). Plants treated with AOA display basal ethylene production without any climacteric rise during senescence. Successful extension of vase life has been displayed by many flowers treated with AOA as holding solutions (Yu *et al.*, 1979; Rattanwisalanon, 2002; Chamani *et al.*, 2005). This can be attributed not only to the fact that AOA prevents the formation of ACC, but also to the ability of AOA to improve water uptake and prevent microbial growth in holding solutions (Ketsa *et al.*, 2001). Furthermore, it has also been reported that AOA blocks the hydrolysis of sucrose to glucose and fructose in senescing tissues, controlling the translocation of sugars from wilting petals to other organs of the flower.

##### 2.6.1.1.2 Aminoethoxyvinylglycine (AVG)

Aminoethoxyvinylglycine (AVG), an analogue of the antibiotic rhizobitoxin, is another example of ethylene production antagonist. It was isolated from a *Streptomyces* strain (Matoo *et al.*, 1979) and was found to be the most potent inhibitor of ACC synthase

amongst vinylglycine analogs (Yang and Hoffman, 1984). The mechanism of inhibition by AVG is similar to that of AOA where it inhibits ethylene production by preventing the conversion of AdoMet to ACC by inhibiting ACCS (Bramlage *et al.,* 1980).

Furthermore it was found that AVG inhibits methionine synthesis by affecting 8-cystathionine activity, an intermediate of methionine in the initial stages of ethylene synthesis. AVG has been widely used in extending vase life and maintaining quality of flowers and fruits. It was found that treatment of the stigmatic surface of *Cymbidium* prior to pollination effectively blocked ethylene production and eliminated pollination induced symptoms (Woltering *et al.,* 1993). AVG also contributes to the maintenance of turgidity of flower petals as it improves water uptake, which is similarly observed in AOA. In fruits, such as peach, inhibition of ethylene production by the application of AVG also resulted in increased firmness and delayed flesh softening (Bregoli *et al.,* 2002). Nevertheless, the effects of AVG can only counter endogenous ethylene effects. Exposure of AVG treated ethylene-sensitive plants with exogenous ethylene will still result in senescence.

#### 2.6.1.2 Ethylene Action Inhibitors

Ethylene action inhibitors suppress the ethylene response by blocking the binding of ethylene to receptors. This is done via a competitive or non competitive manner. For inhibitors such as Norbornadiene (NBD), where inhibition is competitive, continuous presence of this chemical is needed to counteract ethylene effects. However, non-competitive inhibitors such as silver ions need not be present continuously and are usually applied as pulsed solutions. Silver-bond receptors remain in a locked state and disallow conformational change, thus, disallowing the inactivation of the receptor (Serek *et al.,* 2006).

#### 2.6.1.2.1 Silver Thiosulphate (STS)

One of the most widely used inhibitor for ethylene action is silver thiosulphate (STS), a chemical developed as an improved version of using silver ions solely. Earlier experimental work has indicated that silver ions move slowly in the stems of cut carnations. Since silver ions must penetrate to the site of ethylene action, it must thus be converted to a form which can be taken up and transported much more readily than in the ionic form. It was later found that silver moves more readily in stems if it is present as the silver thiosulphate complex (STS) which is formed by the combination of $AgNO_3$ and $Na_2SO_3$ in specific molar ratios (Reid *et al.*, 1980). STS became the preferred choice because of the relatively easy application and low cost per plant. Furthermore, studies conducted showed increased uptake in mobility in plant tissues (Veen, 1983). STS is usually pulsed as pre-treatment for cut flowers and applied as aqueous spray for potted plants. STS has been proven to prolong the vase life and reduce abscission in *Thalictrum* (Hansen *et al.,* 1996), *Alstromeria* (Wagstaff *et al.,* 2005) and *Gladiolus* (Meir *et al.,* 1995).

#### 2.6.1.2.2 1-Methylcyclopropane (1-MCP)

1-MCP is a volatile cyclic olefin, analogous to one of the photolytic composition products of diazocyclopentadine that binds to cellular ethylene receptors (Serek *et al.,* 1994). 1-MCP was developed as an alternative to STS and is used in various industries including cut flowers, potted flowers, bedding, nursery and foliage plants, and in stored fruits and vegetables.

This compound is considered as the inhibitor of choice for most industries as it is odorless and non-toxic at its active concentration (Sisler and Serek, 1999). The antagonism created by 1-MCP is via competitive binding to ethylene receptors. Hence, flowers or fruits may still produce ethylene, but will be unable to response in the presence of 1-MCP.Extremely low concentrations of 1-MCP reduced senescence, induced opening of additional flowers and prevented ethephon-induced flower shattering in *Lupinus Havardii* (Sankla *et al.,* 2000). In the presence of ethylene, the vase life of carnations, *Alstroemeria* and *Anthirrinum* were up to 4 times longer than the untreated flowers (Serek *et al.,*1994).

1-MCP represents the best example of a group of active cyclopropene compounds based on concentration and stability considerations. Once bound to ethylene, 1-MCP will remain attached to receptors before disassociating slowly after a period of time. In carnations, flowers became sensitive to ethylene after 12 days at 25°C and some dissociation is thought to take place (Sisler and Serek, 1999). The eventual response to ethylene in the presence of 1-MCP may also be due to the synthesis of new ethylene receptors overtime. Expression of ethylene genes have been shown to increase with flower senescence (Payton, 1999; Orzaez *et al.*, 1999), and may explain the reduced effectiveness of 1-MCP overtime.

### 2.6.2 The Role of Endogenous Sugar in Flower

Two major arguments have been suggested in the effort of determining the role of sugar in senescence. The first argument suggests that the accumulation of sugar in plants is the direct cause of senescence while the opposing argument believes that sugar starvation is what causes cell death. Evidence which support the first argument is rather weak and is dependent on observations like the hastening of leaf yellowing in cut stems

of a few species such as the *Liliummulti florum and Alstoreium pelegirina.* Similar effects however are not observed in numerous other species (van Doorne, 2004).

On the contrary, there is substantial evidence that sugar starvation is the cause of petal senescence. Similarities in changes in ultra structure, metabolism and gene expression is observed in both senescing and sugar starved petals. Both tissues exhibit mass degradation of starch, proteins, lipids and nucleic acid. In carbohydrate starved rice suspension cells, vacuoles engulf various organelles and portions of the cytoplasm enclosed in vesicles. The membranes remain visible for some time but are eventually digested (Matile and Winkenback, 1971; Yu *et al.,* 1999). Similar changes in ultra structure are also present before visible petal senescence in senescing flowers. Sugar-starved petals exhibit extensive degradation in energy source resulting in the eventual breakdown of lipids and proteins as a substitute in sugar starved maize (*Zea mays*) root tips (Brouquuisse *et al.,* 1998). Upon starvation, expression of genes involved in the breakdown of starch and protein are enhanced which include amylase, protease, and genes involved in the synthesis of amino acids and amides. Treatment of exogenous sugars has been found to suppress expression of both senescence and starvation induced genes such as Asn synthetase in starving asparagus (*Asparagus officinalis*) cells. The expression of protease gene was also delayed by the application of exogenous sugars (Graham *et al.*, 1994; Eason *et al.*, 1997).

Two hypotheses have been suggested in determining the role of sugar starvation in petal senescence. The first hypothesis suggests that sugar starvation may result in premature senescence, independent of a maturation signal while the second argues that sugar starvation is a result of maturation signals (Thimann *et al.,* 1977). However there is yet to be a conclusive hypothesis to determine the dependency of sugar starvation on maturation signals in the process of senescence.

### 2.6.3 The role of Exogenous Sugars in Extending Vase Life

Many studies have reported on the application of sucrose in extending the vase life of cut flowers. For example, Nair and Tung (1980; 1985) have reported the effect of exogenous sugars and cytokinins on delaying orchid senescence in *Oncidium* Goldiana. Paulin and Jamain (1982) and Kaltaler and Steponkus (1976) demonstrated the vase life of cut carnations and roses, respectively, increased following sucrose treatment. The extension of shelf life is attributed to the role of exogenous sugars as food and substrates for respiration in flowers. The presence of exogenous sugars also allows for maintenance of cell wall structure and protein content which would normally be broken down as an alternative energy in the absence of sugar (Ichimura, 1998).

Furthermore, studies have shown that optimum concentrations of sugar in holding solutions increases osmotic concentration, thus improves water uptake by flowers. Although the mode of action is still unclear, the desensitization of ethylene receptors through suppression of expression have been suggested to be caused by sugars. However, those opposing this idea suggest that suppression of receptors genes is not necessary, as this reduction in sensitivity could be caused by the accumulation of carbohydrates solely (Ichimura *et al.,* 2000). Sugars have also been reported to have a

detoxifying effect on ethylene inhibitors, thereby allowing for an inhibiting action on flowers without the toxic side-effect.

## 2.7 BIOTECHNOLOGY FOR ORCHID IMPROVEMENT

Extension of shelf life has been a major target for flower improvement throughout the years. Studies of the ethylene biosynthesis and perception pathway, and the isolation of the related genes have enabled scientists to modify and insert genes that alter shelf life. Two main genetic engineering approaches have emerged targeting the production and perception of ethylene. These approaches consist of anti-sense or sense transformation of *ACCO* and *ACCS*, and the employment of heterologous expression of mutated ethylene receptor genes.

### 2.7.1 Manipulation of Ethylene Biosynthesis Enzyme Genes

The aim of manipulating ethylene biosynthesis genes is to achieve maximum reduction of ethylene production which would result in an extension of flower longevity. Anti-sensing the biosynthesis genes would suppress ethylene production via suppression of ACC production, the precursor for ethylene or by disallowing the conversion of ACC to ethylene. Flowers such as carnation (Savin *et al.*,, 1995), begonia (Einset and Kopperut, 1995), and torenia (Aida *et al.,* 1998) have all been transformed with an antisense version of *ACCO* gene. Transgenic carnations transformed with an *ACCO* gene under the control of the MAC promoter, constructed from elements of the CaMV 35S and *Agrobacterium* nopaline synthase promoter, produced 90% less ethylene than untransformed flowers. The flowers also exhibited a longer shelf life of 9 days compared to only 5 days in untransformed flowers (Savin *et al.,* 1995). Transgenic bogenia managed to live as long as untransformed flowers treated with STS, with a 20-30% reduction in enzyme production (Einsett and Kopperut, 1995). In transgenic

teronia, shelf life was extended 3.5 times more than untransformed flowers. Furthermore, it was also observed that transgenic lines produced more flowers per stem (Aida *et al.,* 1998). Kosugi *et al.,* (2000) employed the transformation of *ACCO1* in the sense orientation to produce transgenic carnations with extended shelf life. This method is thought to prohibit the expression of *ACCO1* via co-suppression in the gyneocium. In potted carnations, in vivo ACC oxidase enzyme activity in leaflets segments of cultured shoots was significantly reduced in plants transformed with *ACCO* in both anti-sense and sense orientation (Kinouchi *et al.*, 2006).

Compared to *ACCO*, manipulation of *ACCS* to produce transgenic flowers with extended shelf life has been less successful. Construction of an effective antisense strategy is made difficult by the multiple forms of this gene within the plant genome (Theologis and Sato, 1998). Moreover, *ACCS* is present only fleetingly just prior to its action, making it less accessible and more difficult to isolate (Stearns and Glick, 2003). Initial attempt of transforming carnations with antisense *ACCS* was successful in extending shelf life, but resulted in the compromise of other characteristics including shorter root system, rendering the flowers less attractive to breeders. When petunia plants were transformed with anti *ACCO1* and *ACCS1* from broccoli, it was found that although both types of transformants exhibited longer shelf life compared to untransformed flowers, the delayed senescence was more pronounced in antisence *BoACCO1* than the antisense *BoACCS1*. Furthermore, transformation with *BoACCO1* was evidently more effective than *BoACCS1* in reducing ethylene production as much lower ethylene production was observed in excised shoots of *BoACCO1* clones (Huang *et al.,* 2007).

### 2.7.2 Manipulation of Ethylene Receptor Genes

Remarkable progress has been achieved since the elucidation of the ethylene transduction pathway in *Arabidopsis* (Bleecker and Schaller, 1996). Studies using *Arabidopsis* as the model plant showed that ethylene receptors act as negative regulators. Therefore, a loss of function mutation would result in constitutive ethylene response as repression of ethylene signalling is impeded. On the other hand, mutation in the binding domain of ethylene receptors would make plants insensitive to ethylene, as the receptors remain "locked" in their active state (Stearns and Glick, 2003). The isolation of *ETR1* from *Arabidopsis* led to the development of chemical-free transgenic plants with prolonged shelf life through the introduction of a mutated *etr1-1* gene. It proved to confer dominant ethylene resistance in tissues where it is expressed, delaying fruit ripening and flower senescence in tomato and petunia plants (Wilkinson *et al.*, 1997). The main concern of this strategy however, is the inability of plants to develop normally, considering ethylene response is also crucial for other facets of plant development.

Transformation of *etr1-1* into petunia, for instance, managed to prolong floral shelf life, but at the same time severely affected the rooting system (Clark *et al.*, 1999). This concern was then addressed with the usage of organ- and tissue- specific promoters that are internally induced by plant development or externally by environmental stimuli. Transformation of *etr1-1* into carnations under the control of its own promoter, a constitutive CaMV35S promoter or an *FBPI* (floral binding protein) managed to extend the shelf life of carnations for at least 6 days, with a maximum of 16 days. This not only exceeds the shelf life of untransformed carnations, but also supersedes the shelf life of carnations treated with ethylene antagonists (Bovy *et al.*, 1999). Transgenic petunia transformed under the control of *FBPI* and AP3 involved in floral development)

promoters achieved 5 times longer shelf life compared to untransformed ones (Cobb *et al.,* 2002).

Another mutated ethylene receptor gene that has shown success in extending shelf life, is the *boers* gene. This gene was isolated from *Brassica oleracea* and was first introduced into petunia (Shaw *et al.,* 2002). Narumi *et al*., (2005) transformed mutated *ERS* genes (one-nucleotide substitution) and obtained transgenic lines with high ability to confer reduced ethylene sensitivity. The *boers* gene was similar to *etr1-1* as it also codes for an ethylene receptor with a non-functional sensor domain, resulting in the inability of the receptor to bind to ethylene. When petunia plants were transformed with the *boers* gene, not only did the flowers exhibit longer shelf life, the flowers were also more turgid, larger and retained pigmentation longer than the non-transgenic plants. The flowers however, were more susceptible to disease. Diminished disease resistance is not exclusive to transgenic petunia, as similar observations have been reported in soybean (Hoffman *et al.,* 1999) and tobacco (Ohtsubo *et al.,* 1999). This calls for the employment of specific promoters that could confine expression, allowing for disease resistance and other ethylene related activities to continue to occur in other parts of the plants (Shaw *et al.,* 2002).