# CHAPTER 8
# GENERAL DISCUSSION

Orchid flowers are noted for their great variety in sizes, colours and shapes. They have interesting and unique structures like a highly modified petal known as the labellum or lip and sometimes a spectacular dorsal sepal. These attractive features require a great deal of energy for development and maintenance and utilize a large proportion of resources (Ashman and Schoen, 1994). Orchid flowers live for a long time and this increases energy demands and expenditure. Orchid flowers such as those of *Cymbidium* and *Phalaenopis* have been reported to stay open and fresh for months. This ensures that flowers remain attractive for long periods and this increases the probability that they will be visited by a pollinator and pollinated (Arditti, 1979). Such large expenditures of energy are justified in terms of evolution because they increase the likelihood of pollination and the survival of species. However, once a flower is pollinated expenditure of energy is no longer necessary and/or justified evolutionarily. There are also orchid species, as for example *D. appendiculum* and *D. crumenatum*, which have short lived (i.e., ephemeral) flowers. In the case of *D. crumenatum* the flowers live for only a day, but flowering is gregarious and the pollinator of this species (a bee) is attracted by fragrance and the multitude of blossoms.

Orchids are economically important flowers that bring significant revenues through both the cut flower and potted plant industries. Investigations of factors which bring about senescence its effects on the orchid cut flower and pot plant industries have had significant practical benefits to the industry. In this thesis, the hybrid *D.* Pompadour was used as the model flower. Although *D.* Pompadour flowers are not propagated through pollination, this hybrid is a fair representation of the parent species because the flowers resemble it to a very large extent. Furthermore, the rapid senescence as well as

distinct degenerative events induced by pollination, which are no different from those in the parent species render this flower a suitable model for studying post-pollination phenomena. The single flower system used in this study is also an effective experimental model for the study of the effects of pollination and treatment solutions on the vase life and quality of pollinated orchid flowers. Individual flowers of similar age provide more consistent results in terms of the changes induced by pollination. In contrast, pollination induced senescence on an entire inflorescence may be affected by the different rates of nutrient distribution amongst all the flowers. Symptoms induced by pollination and removal of pollinia (i. e., emasculation) and damage to flowers during transport and storage are very similar. Therefore findings from research on post pollination phenomena are of importance to those who grow, pack, ship, store and display orchid cut flowers and pot plants.

Data obtained from this study reveal the drastic reduction in longevity of pollinated *D.* Pompadour. Pollinated flowers were fully closed two days after pollination. In contrast unpollinated blossoms closed after 19 days. Progression of pollination-induced senescence symptoms continued after full closure. The first signs of pronounced venation were observed on the $4^{th}$ day and by the $7^{th}$ day the initial indications of browning and necrosis were visible. These five distinct symptoms were used to categorize the senescence process of pollinated *D.* Pompadour into five stages. The changes in the perianth of this flowers show that they undergo similar morphological changes as other orchids such as *A. sesquipedde* where the entire flower closes and the blossom eventually dies. In *A. eburneum* although only the labellum folds, the entire perianth still dies (Avadhani *et al*., 1994).

It is reasonable to assume that such an orderly morphological change must be highly regulated. In this case, the production of ethylene was measured to confirm the role of the gas in regulating pollination-induced senescence. The results showed that a burst of ethylene occurred in pollinated *D.* Pompadour as early as 6 hours after pollination which coincided with the earliest signs of upward petal movement. Similar observations have been reported in orchids where ethylene peak coincided with inward movement or wilting of petals (Arditti, 1979).

The trend of ethylene production in pollinated *D.* Pompadour exhibited the signature pattern of climacteric plants where a peak of ethylene production is followed by a subsequent drop. As in other climacteric plants where ethylene need not be present continuously for senescence to progress, pollinated *D.* Pompadour continued to senesce and showed visual signs of deterioration even after hormone was no longer detected.

Ketsa *et al.,* (1996) postulated that the ethylene production originated from the stigma where ACC from pollen induces ethylene production. Subsequently ethylene is translocated throughout the flower to signal for senescence–related events. Ethylene production in pollinated flowers is also induced by wounding and emasculation. In effect removal of pollinaria causes a wound. Therefore post-pollination ethylene is a wound response. Further observations on pollinated *D.* Pompadour show physiological changes akin to deteriorative events accompanying senescence. These include change in colour, loss in petal thickness, decline in fresh weight, dry weight and reduced water uptake and content. It was also evident that these changes were accelerated by pollination as unpollinated *D.* Pompadour showed limited or no changes. When changes did occur they took place much later.

The data regarding the physiology of pollination induced changes in *D.* Pompadour flowers and research by others show that orchids have evolved deteriorative mechanisms which ensure that segments in pollinated flowers which are no longer biologically necessary are broken down quickly. Avadhani *et al.,* (1994) suggested that some segment in pollinated flowers of many orchids have completed their functions and therefore need not be maintained on the plant. In a few orchids (Some *Zygopetallum* and *Phalaenopsis* species) perianth segments turn green, become photosythic and contribute carbon to developing fruits.

Considering the pivotal role of ethylene in regulating pollination-induced senescence, the application of inhibitors of this hormone can be expected to counter or reduce the effects of pollination. In this thesis, two ethylene inhibitors that antagonize its effects differently ways were applied to *D.* Pompadour flowers; AOA which block ethylene biosynthesis and STS which affects sensitivity to it. Both the inhibitors extended the longevity of the pollinated flowers. Solutions of 0.05 mM AOA and 0.6 mM STS successfully prolonged longevity more than 4 folds. Morphologically, flowers treated with the inhibitors were similar in appearance as unpollinated ones. Other investigators working with other orchids have also reported on the effectiveness of AOA and STS in maintaining shelf life and keeping quality. These included *D.* Sonia (Ketsa, 2004), *D.* Heang Beauty (Chandran *et al.,* 2006) and *Oncidium* Gower Ramsey (Chandran *et al.,* 2007).

Apart from the ability to block ethylene biosynthesis and sensitivity to it these inhibitors also have antimicrobial and antibiocide properties. The acidity of AOA not only inhibits microbial growth but has also been reported to improve water uptake. This was also demonstrated in this thesis because pollinated flowers treated with AOA exhibited improved water uptake compared to the control.

The events that occur during pollination-induced senescence require and a supply of respirable substrates. However, since the pool of such substrates is limited, their eventual depletion would also lead to death of flowers. Therefore, a continuous supply of energy in the form of sugars can be expected to increase flower longevity and quality. Data in this thesis show that although sugars were able to extend vase life of unpollinated flowers, the effects were not pronounced enough to extend for more than two days. As a result, the sugar treatments prolonged vase life of pollinated *D.* Pompadour two fold. This is far less that the extensions brought about the ethylene inhibitors. Similar results were reported for *D.* Sonia in which sucrose treatment did not reduce pollination effects on flowers but prolonged vase life and delaying wilting of unpollinated blossoms (Ketsa, 2004). This was also the case with pollinated *D.* Heang Beauty in which sugar treatments delayed hyponasty by only 1-2 days compared to 5-6 days by AOA (Chandran *et al.*, 2006).

Based on these reports and findings in this thesis, it can be concluded that although sugars may be among the major factors that affect vase life of unpollinated flowers (van Doorn, 1997), they do not have a similar effect on pollinated blooms. Whether or not different ethylene pathways exist in senescence of pollinated flowers and unpollinated flowers have yet to be determined.The findings in this thesis also present an insight into the biochemical changes that take place during pollination-induced senescence. The

results indicate that pollination induces several hydrolytic processes that lead to the eventual senescence of flowers. This is evident by the breakdown of complex molecules including starch and protein and the elevated levels of cell wall hydrolases such as PME, PL and cellulase.

Carbohydrate status has been suggested as one of the major factors that contribute to flower longevity. Furthermore, carbohydrates may even play a role in affecting factors such as ethylene and water relations that also contribute to longevity (Darras *et al.,* 2010). The reduction in starch content parallel with the rise in total sugars is in accord with previous reports for pollinated *Cattleya* (Hsiang, 1951) and *Aerides* sp. (Attri *et al.,* 2008). Similar patterns have also been reported in ethylene-insensitive lilies where withering of flowers coincided with an approximate 50% reduction instarch content (van der Meulen-Muisers *et al.,* 2001). Starch status in this study compliments the morphological changes and longevity in pollinated *D.* Pompadour. In pollinated flowers, reduction in starch was observed whereas in unpollinated flowers higher levels were evident.

An exponential relationship between starch content and longevity has been reported in detached flowers of *Rosa* hybrids where flowers with higher starch levels in tepals were found to have a longer vase life. Pun and Ichimura (2003) postulated that sugars affect flower longevity by not only providing substrates for respirations and adjusting osmotic pressure, but may also be involved in the suppression of ethylene biosynthesis and sensitivity. Opposite trends were reported by Ichimura *et al.,* (2005) who showed that short lived cultivars contain higher starch concentrations than long-lived ones. Although these contradicting reports exist, the fact that pollination-induced senescence is the beginning of the process of ovary development cannot be overlooked. Upon

pollination, the perianth undergoes a change in function from being a sink organ to a source organ. On the other hand, the ovary becomes into a sink organ. Therefore, the theory of starch breakdown and remobilization would be more plausible.

The reduction in starch content is accompanied by the resultant increase in total sugars in pollinated *D.* Pompadour flowers. Reducing sugars seem to play a more important role in post pollination phenomena as exemplified by the elevated levels of reducing sugars as opposed to the declining concentrations of sucrose. This is in agreement with studies carried out on senescing roses (Kaltaler and Steponkus, 1974) and carnations (Nichols, 1973) where reducing sugars rather than sucrose were noted as the main constituents of the sugar pool. Aside from their role as respirable substrates, sugars have been presumed to act as secondary signals during pollination-induced senescence.

In this thesis, the involvement of four cell wall hydrolases; PG, PL, PME and cellulase were investigated in pollinated *D.* Pompadour. Hydrolytic enzymes perform two functions in pollinated orchid flowers. First, by breaking down the integrity of cells and internal structures as well as pigment and other substances they bring about changes in the colour, morphology, olfactory character and general appearance of flowers. This is important because these features play major roles in attracting pollinating vectors. The altered blossoms do not attract pollinators thus preventing, or at least reducing, visits to flowers which would serve no useful purpose and increasing the frequency of visits to unpollinated blooms. Since orchid pollinators can be scarce this can increase seed production (Avadhani *et al.*, 1994). Second, the degradation of macromolecules produces smaller substances which are transported from floral segments which have completed their functions (i.e., perianth segments) to ovaries where pollination induced activities (ovule production which in orchids starts after pollination, zygote

development, seed production, increased respiration) take place (Avadhani *et al.,* 1994). This reutilization of substances and conservation of energy contributes to the survival of orchids (Arditti, 1979, 1994).

The results show that PG and PL increased concomitantly with the upward movement of in pollinated *D.* Pompadour with PG exhibiting a higher level of activity. Both enzymes are responsible for the breakdown of pectin which not only results in structural changes but also in the production of simpler saccharides. Interestingly, PME, another enzyme involved in pectin degradation showed a much lower rate of activity in pollinated *D.* Pompadour. Low PME activity has also been reported in other orchids such as *D.* and *D.* Savin White. In *D. crumenatum*, PME activity was found to peak at the earliest stage of bud development but remained low during senescence, although pectin continued to be solubilized (Yap, 2008). The author postulated that pectin solubilization may be carried out by other hydrolases. In this thesis however, it is difficult to conclude whether or not PME plays a role at any point of flower development prior to senescence as studies were carried out upon pollination. Based on the results obtained in this thesis, it is reasonable to speculate that PME may not play as important a role as PG and PL in pectin solubilization during pollination-induced senescence.

Cellulase, the enzyme involved in the breakdown of cellulose also increased concomitantly with the upward movement of the perianth in pollinated *D.* Pompadour flowers. Similar observations have been reported in *D. crumenatum* (Yap, 2008) and daylilies (Panavas and Rubeinsten, 1998). Breakdown of cellulose has been reported to play a role in the remobilization of sugars in senescing flowers. As was discussed earlier, pollination results in the conversion of perianth segments into source organs.

Therefore it is of no surprise that cellulase activity has been observed to increase in this study. The collective action of these cell wall hydrolase culminates into the total disintegration of cell walls and the collapse of parenchyma layers. This in turn results in the diminished protection for the cell membrane.

Electrolyte leakage is indicative of loss of membrane integrity in membrane. In this thesis, relative conductivity was measured to determine electrolyte leakage which reflects membrane permeability and integrity. Pollinated *D.* Pompadour exhibited a large increase in electrolyte leakage at the advance stages of senescence. This coincides with visual symptoms such as necrosis and severe venations were observed. The observation shows an upward trend that reflects increased deterioration of the flower. These observations point to a classic feature of petal senescence which is observed in many senescing flowers including carnations, roses and the short-lived morning glory. In pollinated flowers treated with ethylene inhibitors, the trend for cell wall hydrolase activity and electrolyte leakage mimicked that in unpollinated flowers. This further establishes the role of ethylene in regulating biochemical changes during pollination induced senescence.

As observed in all senescing flowers, aging pollinated *D.* Pompadour also exhibits the universal response of protein degradation. Breakdown of total protein may also serve as an alternative source of energy (van Doorn, 1997) and as a remobilization of nutrient for the developing embryo (Chapin and Jones, 2007). The sum total of protein content is a result of both degradation and synthesis of soluble and insoluble proteins. In this thesis, the data present an insight into the different metabolic processes associated with diverse proteins. Pollination-induced senescence in *D.* Pompadour was found to be preceded by a decrease in water insoluble proteins. Water soluble proteins were relatively

unaffected. Similar observations were reported in *D.* Khao Sanan (Lerslerwong *et al.,* 2009) and *Geraldon* Waxflowers (Olley, 1996). It was postulated that water insoluble proteins are mainly localized in the membrane (Hooshdaran *et al.*, 2004). Thus the degradation of the membrane during pollination-induced senescence as demonstrated by leakage would also mean that proteins attached to the membrane are affected as well. Since insoluble proteins are made up of enzymes, it is possible to assume that these enzymes are being synthesized constantly. They catalyze the existing and ongoing biochemical processes during senescence. As noted in previous chapters, treatments with AOA and STS were also effective in fostering protein retention. Sugars, although not as effective also managed to reduce protein degradation. In flowers of *Sandersonia* and broccoli florets, treatment with sucrose delayed the induction of cystein protease transcription. Coupe *et al.,* (2003) suggested that sucrose may be involved in the regulation of proteases at a transcriptional level.

In order to obtain an overview of the protein changes that occur during pollination induced senescence, 1D SDS PAGE was carried out. Results show that three polypeptides were regulated throughout the process. They were identified as a PR-like protein, SAM synthase and seed storage protein. Amongst the three proteins, the most abundant was the PR-like protein followed by SAM synthase. Seed storage protein was expressed in the smallest amounts. The increase of PR-like polypeptide following pollination (which was highest compared to the rest of the flowers) demonstrates the necessity for a defence response following pollination.

While PR proteins have been widely established in terms of pathogen induction, relatively few studies have been carried out to establish the regulation of PR proteins during plant senescence. The findings in this study suggest the intrinsic dual function of the PR proteins of not only responding to pathogenic attacks, but also in ensuring successful seed development. Given the physical changes and nutrient remobilization that occur following pollination, developing ovaries are highly susceptible to environmental stress and pathogen attacks. Upregulation of PR protein following pollination of *D.* Pompadour is a response to this condition and acts as a pre-emptive mechanism to deal with the environment stress and pathogens. Other studies corroborate the findings in this thesis. In a number of flowers where senescence follows pollination, proteomic and genomic studies reveal that defence proteins are the major proteins being upregulated (Lotan *et al.*, 1988; Day *et al.*, 1998; Bai *et al.*, 2008).

An intriguing question would be whether the PR proteins involved in pathogen induced defence response are the same as those regulated by ethylene produced following pollination. In 1978 Heslop-Harrison speculated that the response towards the intrusion of fungal hyphae in race-specific pathogenicity may have even evolved from angiosperm pollen compatibility systems. This suggests that a gene involved in the normal process of reproduction may have adapted a function in plant defence.

The 27 kDa polypeptide observed in the protein profiles was identified as SAM synthase. Pollination of *D.* Pompadour flowers resulted in an increase in the polypeptide. This observation is not in line with the well accepted fact that ACC, not SAM is the rate limiting substance in ethylene biosynthesis. In fact it was postulated that only a small amount of SAM contribute to the burst in ethylene production. A number of studies support this view with results that show SAM synthase levels

independent of ethylene concentrations. Nevertheless, in mature kiwi fruits (Whittaker *et al.,* 1997) and tobacco flowers (Roeder *et al.,* 2009), the expression of SAM synthase were induced by high ethylene concentrations.

It could be suggested that regulation of SAM synthase by high concentrations of ethylene may be part of the methionine salvage pathway. During high ethylene production levels such as after pollination, SAM is rapidly converted to ACC which leads to ethylene formation. Under this circumstance, the steady state of SAM is disrupted. It is therefore reasonable to asume that the expression of SAM synthase is required to maintain SAM levels. In transgenic tomatoes, the continuous degradation of SAM synthase resulted in a dramatic decrease in ethylene production (1994). The regulation of SAM synthase by ethylene during pollination in *D.* Pompadour is further supported by the observation that AOA suppressed the increase in SAM synthase.

In tobacco a third of SAM synthase levels in the flower was detected in the stigma and style, the parts of the flower where high ethylene synthesis occurs after pollination (in orchids the column or gynostemium is the result of fusion between style, stigma and stamens; the rostellum may be of stigmatic origin). It could be assumed that the high levels of SAM synthase present in the stigma and style initiate ethylene evolution before translocation takes place in other parts of the flower (Nadeau and O' Neill, 1998).

A possible prolamin, a seed storage protein was identified as the third polypeptide which had a size of 15kDa. However, unlike the other two polypeptides, this protein was not produced in abundance in all flowers. This may be due to a number of reasons First, since orchid seeds are the smallest of all seeds, utilization of energy from storage may not as high as by bigger seeds. Second, during early stages of germination nutrients are obtained via mycorrhizal symbiosis. This suggests a reduced dependency on seed reserves. Nevertheless, an increase was still observed in pollinated flowers albeit not as high as PR proteins and SAM synthase. We could safely suggest that development of the embryo would still require some upregulation of storage proteins.

This thesis also deals with the isolation and the characterization of genes involved in the ethylene biosynthesis pathway and ethylene receptor perception pathways. Experiments conducted resulted in the isolation of *ACCO, ACCS, ETR1, ERS1* and *ERS2*. Both *ACCO* and *ACCS* were found to be similar in size to most of the complete *ACCO* and *ACCS* sequences deposited in the databank and contain all the functional domains that characterize the genes. The three receptor genes are partial genes, but nevertheless, contain the signature domains of ethylene receptors. The need to isolate and characterize these genes lies in the demand for a chemical free strategy of extending vase life in flowers. Identifying these genes is the first step of further manipulation using biotechnological methods.

The advent of molecular tools has given birth to genetically modified flowers that are able to circumvent the effects ethylene. The manipulation of *ACCO* and *ACCS* in transgenic carnations resulted in flowers with delayed senescence as a direct result of inhibited ethylene production. However, these flowers still succumbed to exogenous ethylene (Chandler *et al.*, 2007). This problem was overcome by the overexpression of

mutant ethylene receptor that managed to achieve both goals; delaying senescence and overcome sensitivity to ethylene (Milbus *et al.,* 2009; Srikandarajah, 2007). Both strategies of manipulating enzyme genes and receptor genes were applied in carnations where field tests and intercontinental transport studies showed proved stability. Recent reports have also detailed the use of *Arabidopsis* mutant etr1-1 gene in tomato fruits (Gallie, 2010) and petunia (Shibuya, 2004). This broad application was a success as the fruits displayed slower fruit softening and the flowers demonstrated extended vase life both unpollinated and pollinated.

The strategies seem promising, but still have some drawbacks in terms of the additional effects of interfering with the ethylene pathway. Since ethylene is multi-purpose hormone, suppressing the perception resulted in developmental effects which included (among others), poor root formation, reduced seed production and less efficient seed germination (Celvenger *et al.,* 2004). In 2002, Shaw *et al.,* successfully produced ethylene insensitive *Petunia* using a mutated *ERS* homologue isolated from *Brassica oleracea* (boers). The results from that study resembled a previous one, notwithstanding higher mortality rate due to increased susceptibility to fungal diseases. The challenge therefore, is to develop strategies that could circumvent specific pathways. It was suggested that tissue specific promoters were utilized to avoid the unintended developmental effects. This recommendation was taken up when Bovy *et al.,* (1999) who used a flower-specific promoter from *Petunia* (*fbp1*) to transform carnation with *etr1-1*. The resulting plants showed strong insensitivity to ethylene without the unwanted side effects of earlier experiments, though not all agronomical aspects were investigated. The isolation of receptor genes from *D.* Pompadour flowers in this thesis therefore meets a crucial need of developing tissue and stage specific mutant genes that would deal with vase life problems and allow for normal plant development to continue.