# CHAPTER 5
# CELL WALL HYDROLASES AND MEMBRANE LEAKAGE IN POLLINATED *DENDROBIUM* POMPADOUR FLOWERS

## 5.1 INTRODUCTION

The cell wall is a specialised and complex structure, which exists in all plants to serve various important functions. The structure is rigid enough to maintain membrane structure (Paliyath and Droillard, 1992) which acts among others as a protective barrier against pathogens, water loss and mechanical stress (Cosgrove 1999; Carpita and McCann 2000) yet dynamic enough to expand and contract to allow plant growth. The primary cell wall is a complicated matrix made up polysaccharides, proteins and a number of phenolics (Brummell 2006; Liepman *et al.*, 2007). Cellulose, the most abundant polysaccharide found in plants, is also the principle constituent that forms the rigid, structural component of the cell wall (Carpita and McCann 2000). Cellulose microfibrils are composed of 1,4-β-D-glucan chains assembled together by extensive hydrogen bonding, resulting in long, rigid, inextensible fibres. A major assembly of the cell wall is also made up of pectin, a class of polysaccharides that can be linear or branched, highly hydrated and rich in D-galacturonic acid residues. Cell wall modification may be affected by pectins through regulation of enzyme accessibility to substrates as pectins controls wall porosity (Brummell, 2006).

Pollination induced senescence is a programmed cell death which is often associated with alterations in cell wall structure, membrane degradation, remobilization of protein and nucleic acid laddering (Rubinstein, 2000). Interestingly, petal cells often undergo profound changes in size and shape prior to and during senescence, indicating that constituents of the wall are being altered. Where wilting or withering is the final "death" symptom, macromolecules, often those that form the cell wall structure are degraded

into mobile compounds. However, the degree of cell wall degradation seems to be species-dependant. For example, in *Sandersonia* flowers, desiccated flowers still maintain their bell shape (O'Donoghue *et al.,* 2002, 2005) while in *Ipomoea* and *Iris,* considerable parts of the walls of mesophyll cells become degraded (Winkenbach and Matile 1970; van Doorn *et al.,* 2003). Modifications to the cell wall during senescence can be attributed to concerted effort from cell wall hydrolases that affect cell wall assembly and flower senescence.

Although extensive literature can be found on cell wall hydrolase activity in ripening and softening of fruits (Fischer and Bennett, 1991), the same cannot be said about flower senescence. Very little attention has been given to the role of cell wall hydrolases in flower senescence, even less has been studied on cell wall modification in pollination induced senescence. In this chapter the activity of four cell wall hydrolases during pollination induced senescence in *D.* Pompadour flowers were investigated.

The enzymes are polygalacturonase (PG), pectate lyase (PL), pectin methylesterase (PME) and cellulase. PG, PL and PME are the enzymes responsible for the modifications on pectin while cellulase acts on cellulose, the most abundant polysaccharide found in plants. Conceivably, these enzymes are present in all flowers; however, the extent of activity during pollination induced senescence has yet to be elucidated.

Disruption of cell wall also leads to the disruption of membrane integrity, a constant feature in plant senescence and has been linked to changes in turgor and ion leakage Suttle and Kende , (1980) presented evidence of increase in membrane permeability during flower senescence. Phospholipids breakdown was reported to be of great

significance during membrane disruption (Ferguson and Simon, 1973; Borochov *et al.*, 1997). Cell wall and membrane disruption translates into poor flower quality; loss in fresh weight, loss of turgor and wilting or withering of the perianth. As pollination induced senescence is a climacteric process, regulated and hastened by senescence, ethylene inhibitors have been used to stop ethylene signalling thus delaying the effects on the cell wall and membrane.

Sugars, too have long been used as treatment solutions to enhance longevity and improve flower quality. Some studies have also reported that sugars may also positively affect flowers via interaction with ethylene (Leon and Sheen , 2002). Considering the effectiveness of sucrose, glucose, AOA and STS in extending shelf life and maintaining quality of pollinated *D.* Pompadour as established in the first chapter of this study, this chapter will now look into the effects that the treatment solutions have on cell wall hydrolases and membrane leakage.

This chapter therefore aims to investigate the changes in cell wall hydrolase activity and the effectiveness of treatment solutions in delaying or reducing their effects in pollinated *D.* Pompadour flowers.

## 5.2. MATERIALS & METHODS

### 5.2.1. Plant Material

*Dendrobium* Pompadour orchids aged 10-15 days after anthesis were harvested from the University of Malaya Glasshouse and brought to the Postharvest Biotechnology Laboratory. Flowers were cut at the pedicel under water, pollinated and kept in vials containing distilled water or treatment solutions. Treatment solutions which are 2% (w/v) sucrose, 4% glucose (w/v), 0.05 mM AOA and 0.6 mM STS were prepared.

### 5.2.2 Preparation of Sample

Total proteins were extracted by adding 8g of orchid powder (ground using a mortar and pestle in liquid nitrogen) to 16 mL of a modified Kanellis *et al*., (1989) protein extraction buffer containing 50mM Tris-HCl, 0.5M NaCl, 10mM 2-mercaptoethanol, 10μM leupeptin, 1mM DTT, 1mM EDTA, 10% (v/v) glycerol and 0.5% (v/v) triton X-100. The mixture was then vortexed thoroughly, left on ice for 10 minutes and then centrifuged at 25,000 x g for 30 minutes at $4^0$C in a Sorvall RC5C refrigerated centrifuge. The supernatant filtered through one layer of miracloth and used for the subsequent enzyme assays.

### 5.2.3 Determination of Polygalacturonase Activity

PG assay was carried out according to Pathak and Sanwal (1998). 0.2 ml of 0.2M sodium acetate, 0.1 ml of 0.2M sodium chloride and 0.4ml enzyme extract were added into a test tube. 0.3ml 1% (w/v) PGA substrate was then added. The mixture was left for one hour at 37ºc. Subsequently 1ml of DNS was added to stop the enzyme action. The mixture was placed in boiling water for 5 minutes. Next the mixture was taken out from boiling water and 0.4ml of 40% (w/v) Rochelle salt solution was added. Then the mixture was cooled under running water. The absorption was measured using a

spectrophotometer at a wavelength of 540nm. Calculation of enzyme activity was measured against the standard curve of galacturonic acid.

### 5.2.4 Determination of Pectate lyase Activity

The method for PL assay was carried out as described by Moran *et al*., (1968).1.8 ml 4 mM sodium acetate, 0.9 ml 1% (w/v) PGA and 0.3 ml enzyme preparation were added in a test tube. A tube containing the sample blank was prepared by replacing 0.3ml sample with 0.3 ml of sterile distilled water. The reaction mixture was immediately incubated at 37°c for 30 minutes. Then, the mixture was immediately incubated in a boiling water bath for two minutes to stop the enzyme reaction. Absorbance for the sample was measured at a wavelength of 235 nm.

### 5.2.5 Determination of Pectin Methylesterase Activity

The method for PME determination was adopted from Lohani *et al*., (2004). The homogenate was adjusted to pH 7.5 with NaOH. In a 3 ml cuvette, 1 ml of 0.01% (w/v) pectin solution, 0.2 ml 0.15M NaCl, 0.1 ml bromothymol blue, 0.2 ml distilled water and 0.1 ml homogenate were added. Cuvettes were shaken gently and the absorbance was measured using a spectrophotometer at 620nm wavelength immediately and measured again after three minutes. Calculation for the activity was carried out against the standard curve of galacturonic acid drawn as described by Hagerman, (1986).

### 5.2.6 Determination of Cellulase Enzyme Activity

Cellulase assay was carried out according to the method by Dong *et al.,* (1992). 50 mg of cellulose was added to 4 ml of enzyme solution and incubated at 35ºc, with constant agitation of 390 rpm for 16 hours. After incubation, samples were placed in boiling water to stop the enzyme reaction for 10 minutes. Then DNS assay was carried out to measure the amount of reducing sugar present in the sample by adding 1 ml of DNS solution and 1 ml of sample solution. The mixture was then placed in boiling water for five minutes. Then 0.4ml of 40% (w/v) Rochelle salt solution was added and the samples were cooled under running tap water. Finally absorbance readings for all the samples were determined at a wave length of 540 nm. Calculation of enzyme activity was carried out against a glucose standard curve.

### 5.2.7. Measurement of Membrane Leakage

Measurement of membrane leakage was modified based on the method by Xu and Hanson, (2000). Flowers of approximately the same weight (1.5 g) was cut off from the pedicel and placed in a conical flask containing 50 ml of sterile deionised water (SDW). This mixture was placed on an orbital shaker and incubated for one hour. The sample was then tested for sample conductivity. Then, the SDW was discarded and replaced with 50 ml of fresh SDW. Samples were subsequently boiled for five minutes. This was then measured as subtotal conductivity. Membrane leakage is represented by the relative conductivity, which was calculated as sample conductivity divided by total conductivity (the sum of sample conductivity and subtotal conductivity).

## 5.3 RESULTS

PG (Fig 5.1) activity of pollinated flowers held in distilled water showed a pronounced increase beginning 2 days after pollination and peaked 6 days after pollination with an activity of 23.6 µmol $h^{-1}g^{-1}$. PG activity reduced thereafter and settled at 21.3 µmol $^{-1}h$ $g^{-1}$ 8 days after pollination. Unpollinated flowers on the other hand remained unchanged until 4 days, where an increase was observed. This increase continued until the end of the experiment where PG activity in unpollinated measured was 8.4 µmol $h^{-1}g^{-1}$.Flowers held in 4 % glucose and 2% sucrose were rather similar in terms of pattern and rate of activity. Flowers held in both solutions increased 2 days after pollination to a range of 8-11 µmol $h^{-1}g^{-1}$. Flowers held in both the sugar treatments continued to increase and reached a maximum of 14.1 and 14.8 µmol $h^{-1}g^{-1}$ at the end of the experiment. 0.05 mM AOA and 0.6 mM STS were most effective in delaying and surpressing PG activity in pollinated flowers. PG activity remained unchanged in flowers held in these two treatments 2 days after pollination. An increase was observed 4 days after pollination (6.8-7.1 µmol $h^{-1}g^{-1}$) and continued to reach a maximum of 10.2 µmol $h^{-1}g^{-1}$ for flowers held in 0.6mM STS and 11.7 µmol $h^{-1}g^{-1}$ for flowers held in 0.05 mM AOA.

Comparison of the maximum PG activity in all flowers showed highest activity in pollinated flowers held in distilled water (23.6 µmol $h^{-1}g^{-1}$), followed by flowers held in 2% sucrose (14.8 µmol $h^{-1}g^{-1}$), 4% glucose (14.1µmol $h^{-1}g^{-1}$), 0.6 mM STS (11.7µmol $h^{-1}g^{-1}$), 0.05 mM AOA (10.2 µmol $h^{-1}g^{-1}$) and unpollinated flowers held in distilled water (9.4µmol $h^{-1}g^{-1}$).

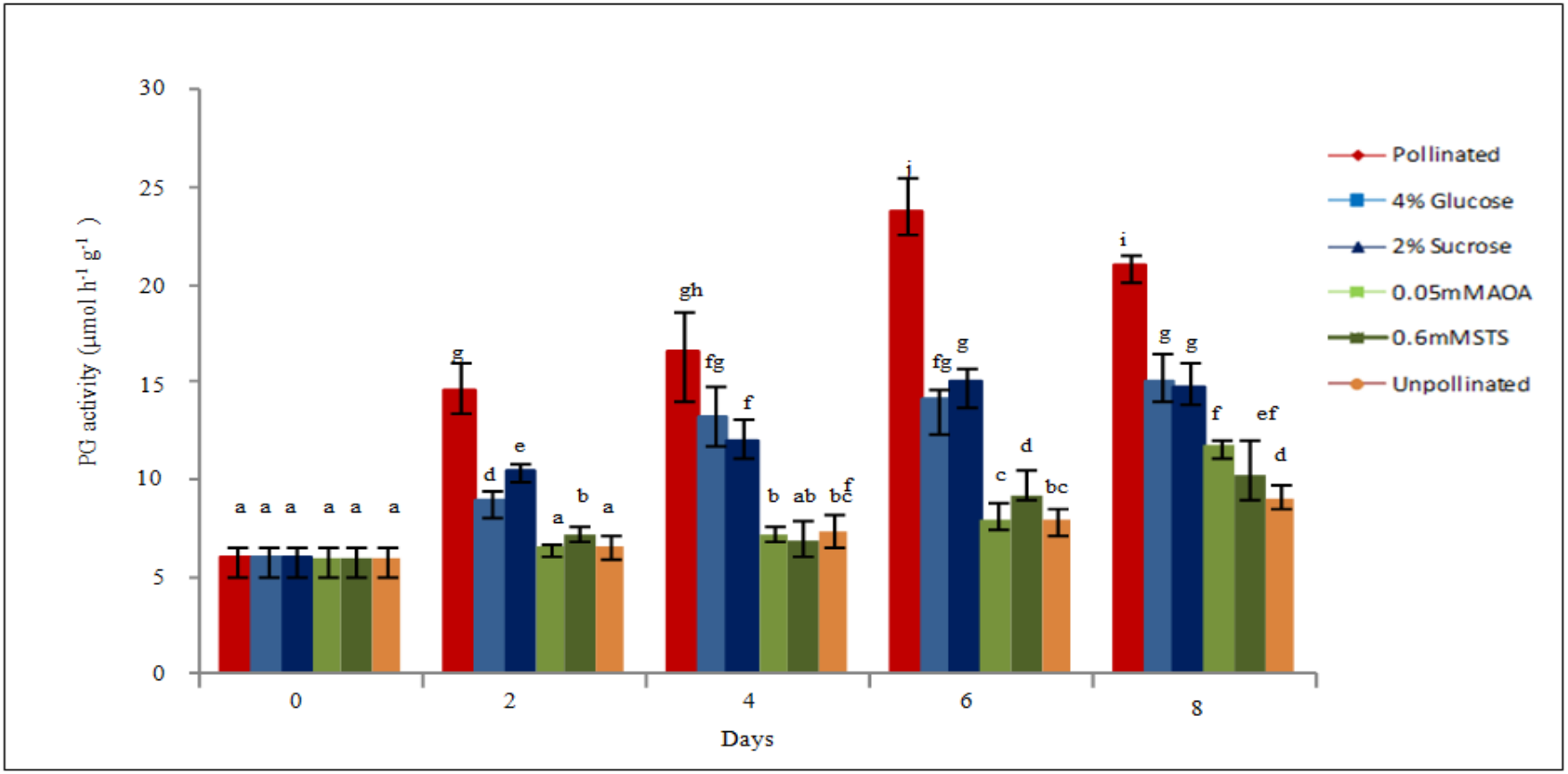


**Fig 5.1 Polygalacturonase (PG) activity of unpollinated, pollinated and treated *D.* Pompadour flowers**. Each value is the mean ± SE ($n$ = 9), and values with different letter(s) are significantly different according to the Duncan's Multiple Range Test ($P < 0.05$).

Overall, pectate lyase activity (Fig 5.2) was lower compared to PG activity in *D.* Pompadour flowers. Significant increase in PL activity was evident in pollinated flowers held in distilled water at the earlier stages of the experiment. PL activity of pollinated flowers held in distilled water increased from 3.4 µmol $h^{-1}g^{-1}$at the beginning of the experiment to 4.8µmol $h^{-1}g^{-1}$ 2 days after pollination. This was then followed by a subsequent increase until the end of the experiment with a PL activity of 6.2µmol $h^{-1}g^{-1}$. In contrast, in unpollinated flowers, PL activity remained unchanged at 3.4 µmol $h^{-1}g^{-1}$ until 4 days, increased to 3.8 µmol$h^{-1}g^{-1}$ on the $6^{th}$ day and remained the same until the end of the experiment.

0.05 mM AOA was most effective in surpressing PL actitvity as it remained unchanged throughout the experiment. PL activity in flowers held in 0.6 mM STS remained unchanged until 8 days after pollination where an increase of 4.2 µmol $h^{-1}g^{-1}$ was measured. PL activity was not as effectively surpressed by the sugar treatments. For flowers held in 2% sucrose, PL activity gradually increased 2 days after pollination (3.8µmol $h^{-1}g^{-1}$) to 6 days after pollination (4.3µmol $h^{-1}g^{-1}$). Therefter, PL activity decreased to 3.5 µmol $h^{-1}g^{-1}$. As for flowers held in 4% glucose, PL activity increased 2 days (4.2 µmol $h^{-1}g^{-1}$) to 4 days (4.5µmol $h^{-1}g^{-1}$) after pollination, decreased 6 days (4.1µmol$h^{-1}g^{-1}$) after pollination and then increased again at the end of the experiment (4.9µmol $h^{-1}g^{-1}$).

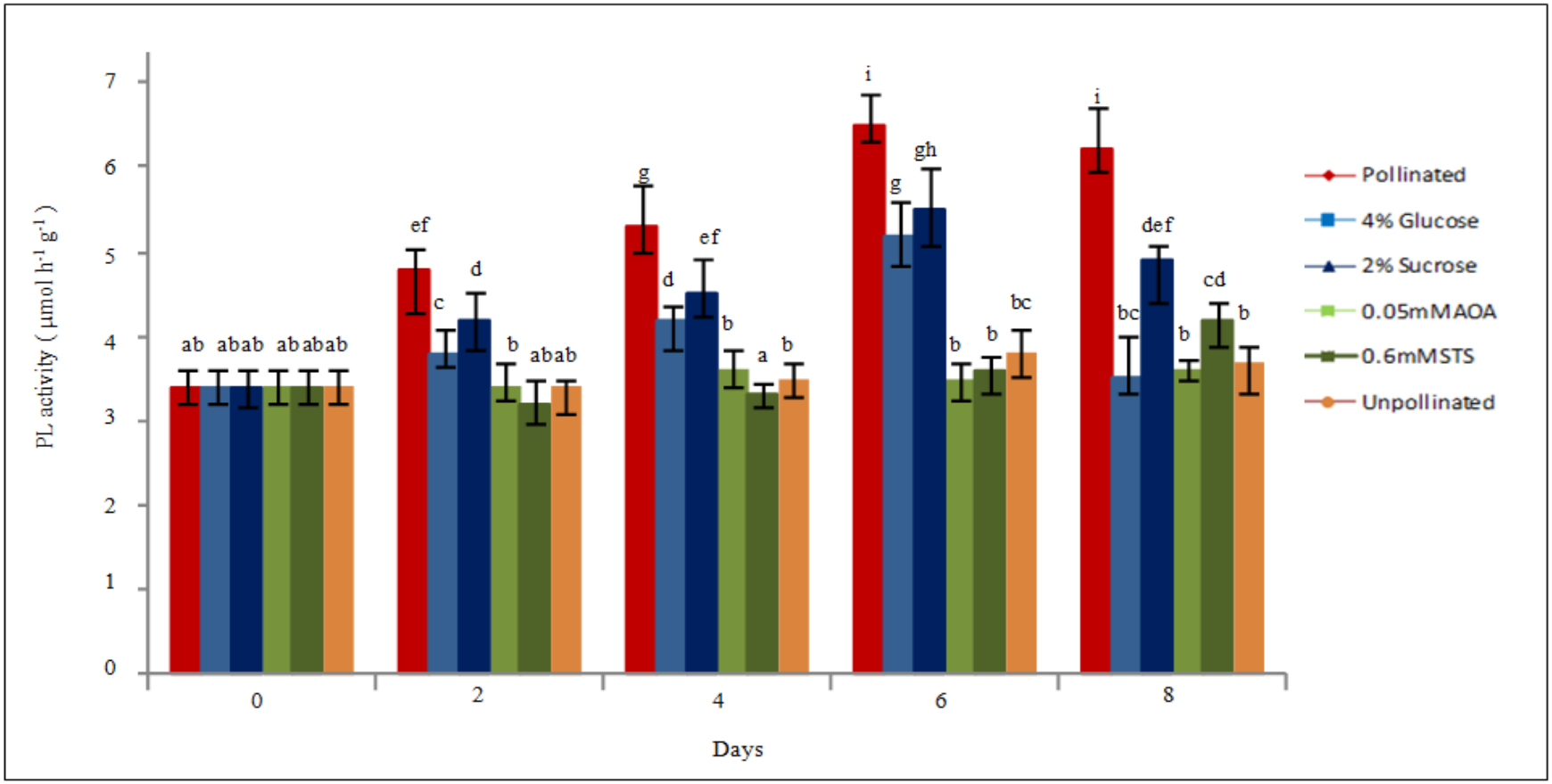


**Fig 5.2 Pectate lyase (PL) activity of unpollinated, pollinated and treated *D.* Pompadour flowers**. Each value is the mean ± SE ($n$ = 9), and values with different letter(s) are significantly different according to the Duncan's Multiple Range Test ($P < 0.05$).

Amongst the three pectin-related hydrolases, pectin methylesterase (PME) was the least significant in *D.* Pompadour flowers as the activity was within a range of 0.55-0.70 µmol $h^{-1}g^{-1}$ (Fig 5.3) . In all the flowers except for pollinated flowers held in distilled water, PME activity remained unchanged. Where changes were detected, increase or decrease in activity was very small.

In pollinated flowers held in distilled water PME activity exhibited a significant increase 6 days after pollination with an activity measured at 0.68 µmol $h^{-1}g^{-1}$ compared to 0.65µmol $h^{-1}g^{-1}$ at the beginning of the experiment. This value was the highest recorded amongst all the samples throughout the experiment. Thereafter, PME activity in pollinated flowers held in distilled water reduced to 0.55 µmol $h^{-1}g^{-1}$. This value was also the lowest activity recorded amongst all the samples throughout the experiment.

In unpollinated flowers, pollinated flowers held in 2% sucrose, 0.05 mM AOA and 0.6 mM STS, changes were insignificant. Negligable decrease or increase was observed in the aforementioned samples throughout the experiment. In pollinated flowers held in 4% glucose, however, a significant decrease was measured on the $6^{th}$ day (0.58µmol $h^{-1}g^{-1}$). Thereafter PME activity in those flowers increased, though not drastically to 0.61µmol$h^{-1}g^{-1}$.

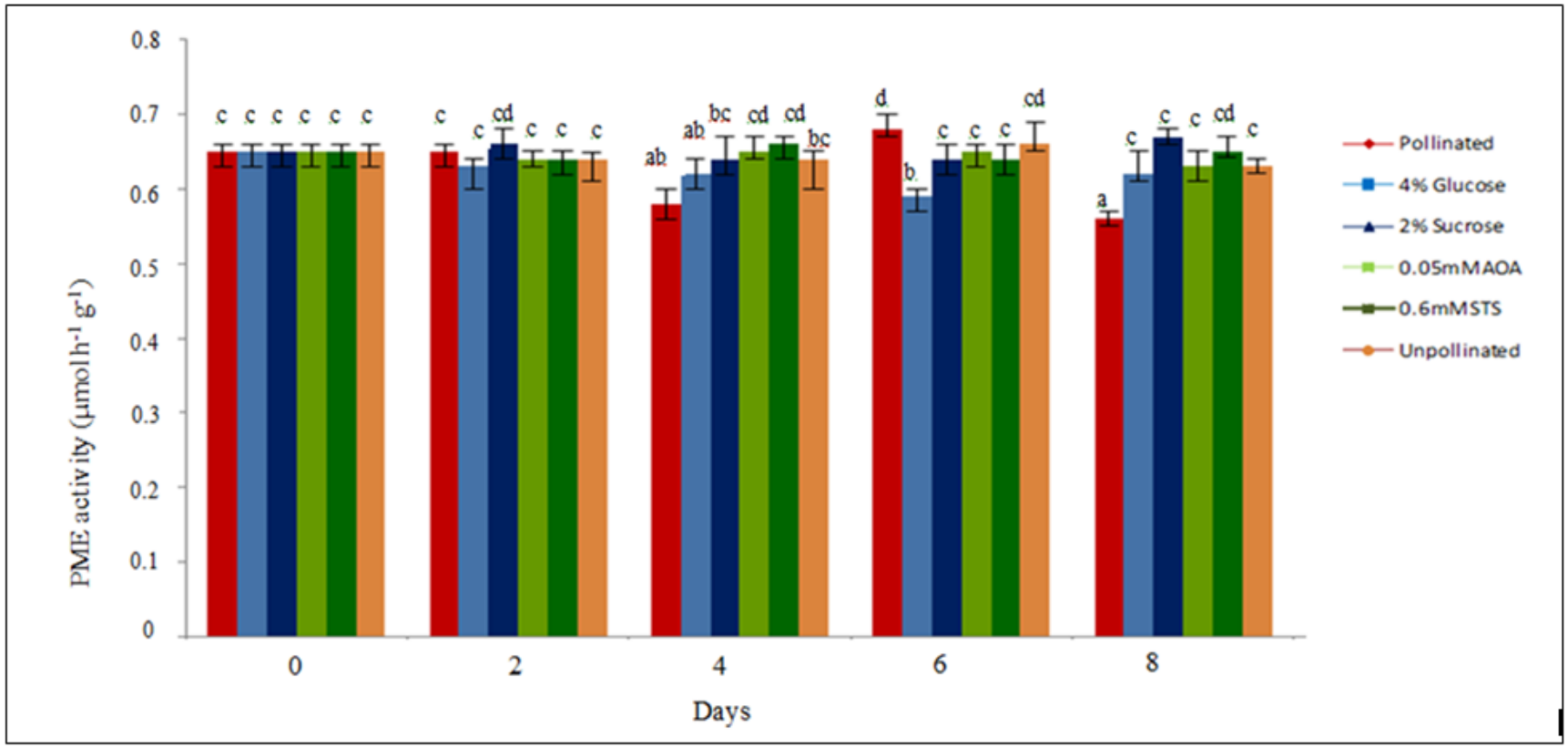


**Fig 5.3 Pectin methylesterase (PME) activity of unpollinated, pollinated and treated *D.* Pompadour flowers.** Each value is the mean ± SE ($n$ = 9), and values with different letter(s) are significantly different according to the Duncan's Multiple Range Test ($P < 0.05$).

The pattern of cellulase activity (Fig 5.4) was similar to that of PG activity in pollinated *D.* Pompadour flowers as changes in activity began 2 days after pollination and subsequently continued to increase thereafter. The rate of activity though is approximately half of the PG activity. Cellulase activity in pollinated flowers was 4.1µmol $h^{-1}g^{-1}$ at the beginning of the experiment, increased to 6.4µmol $h^{-1}g^{-1}$ 2 days after pollination and peaked to 15.3µmol $h^{-1}g^{-1}$ at the end of the experiment. In unpollinated flowers, no significant change was observed in cellulase activity until day 4 where cellualse activity was measured at 4.8 µmol $h^{-1}g^{-1}$ . A significant, though not drastic increase was measured therefter. At the end of the experiment cellulase activity in unpollinated flowers was 6.3 µmol $h^{-1}g^{-1}$ . This is less than half of the activity measured in pollinated flowers.

In all flowers held in treatment solutions, cellulase activity was lesser than that in pollinated flowers held in distilled water. The rate of cellulase activity in flowers held in 0.05 mM AOA and 0.6 mM STS similar to that of unpollinated flowers. 4 days after pollination, cellulase activity in those treatments measured at 5.2 and 5 µmol $h^{-1}g^{-1}$ . At the end of the experiment the enzyme activity for both the treatments was 7.3 and 8 µmol $h^{-1}g^{-1}$ respectively. On the other hand, a more pronounced increase in cellulase activity was measured in flowers held in 4% glucose and 2 % sucrose. By the end of the experiment cellulase activity in flowers held in 4% glucose was 10.4 µmol $h^{-1}g^{-1}$ and 11.8 µmol $h^{-1}g^{-1}$ in 2 % sucrose.

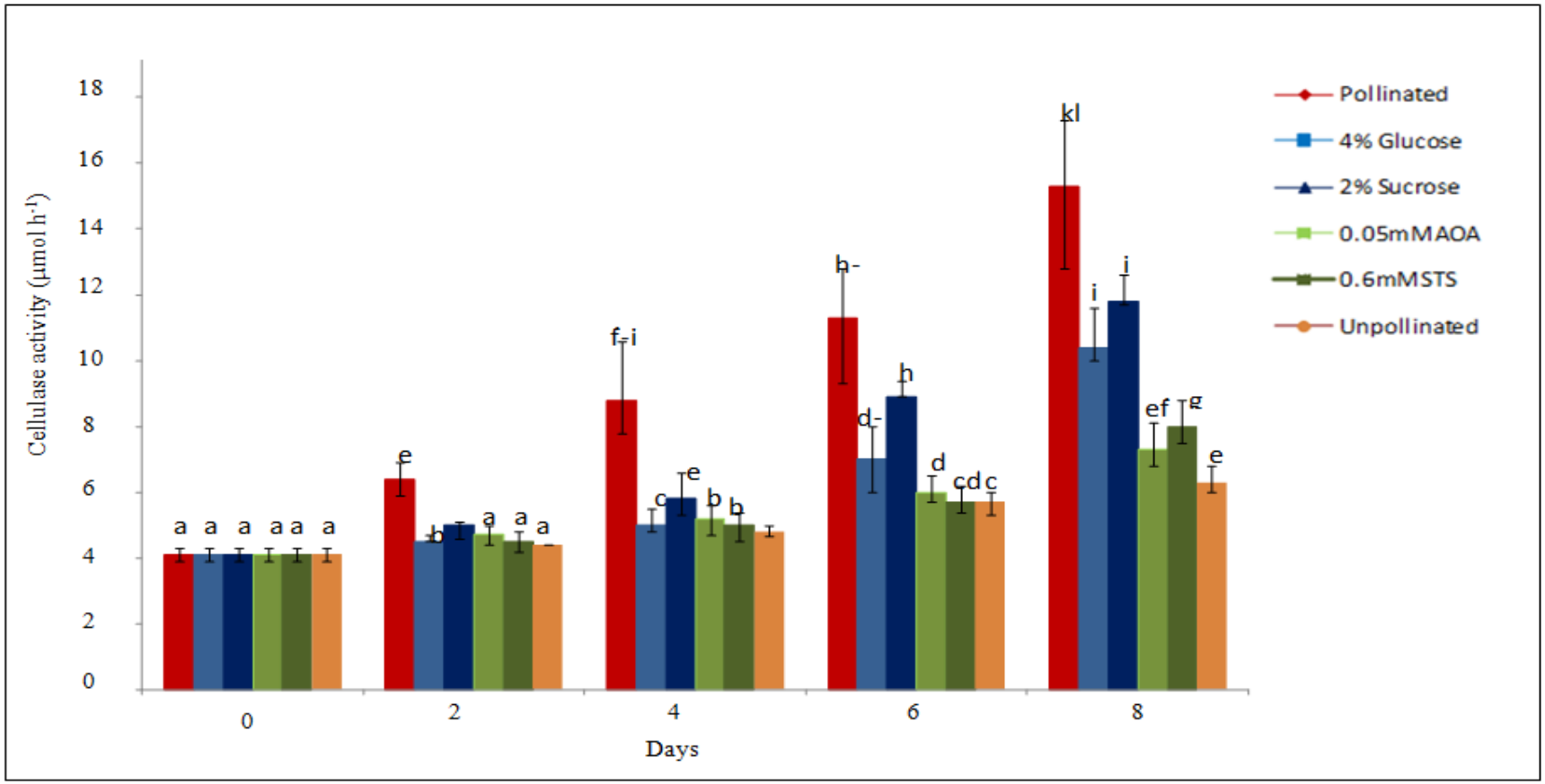


**Fig 5.4. Cellulase activity of unpollinated, pollinated and treated *D.* Pompadour flowers**. Each value is the mean ± SE ($n$ = 9), and values with different letter(s) are significantly different according to the Duncan's Multiple Range Test ($P < 0.05$).

Membrane leakage was more pronounced in pollinated flowers compared to unpollinated flowers. A significant increase in membrane leakage was detected as early as 2 days after pollination and continued thereafter. By the end of the experiment, membrane leakage in pollinated flowers was 0.42. In contrast, changes in membrane leakage was insignificant in unpollinated flowers. At the end of the experiment, unpollinated flowers recorded a value of 0.17 in membrane leakage.

Treatment solutions of 0.05 mM AOA and 0.6 mM STS were very effective in reducing the rate of membrane leakage. A small increase in membrane leakge for flowers held in both solutions was detected 4 days after pollination. By the end of the experiment, pollinated flowers held in 0.05 mM AOA and 0.6 mM STS recorded membrane leakage of 0.19 and 0.18 respectively. On the other hand, the sugar solutions were not as effective in reducing membrane leakage in pollinated flowers. Membrane leaakge was recorded at 0.19 for 4% glucose and 0.25 for 2% surose on the $4^{th}$ day. By the end of the experiment membrane leakage for flowers held in both the sugar solutions was 0.28 and 0.33 respectively.

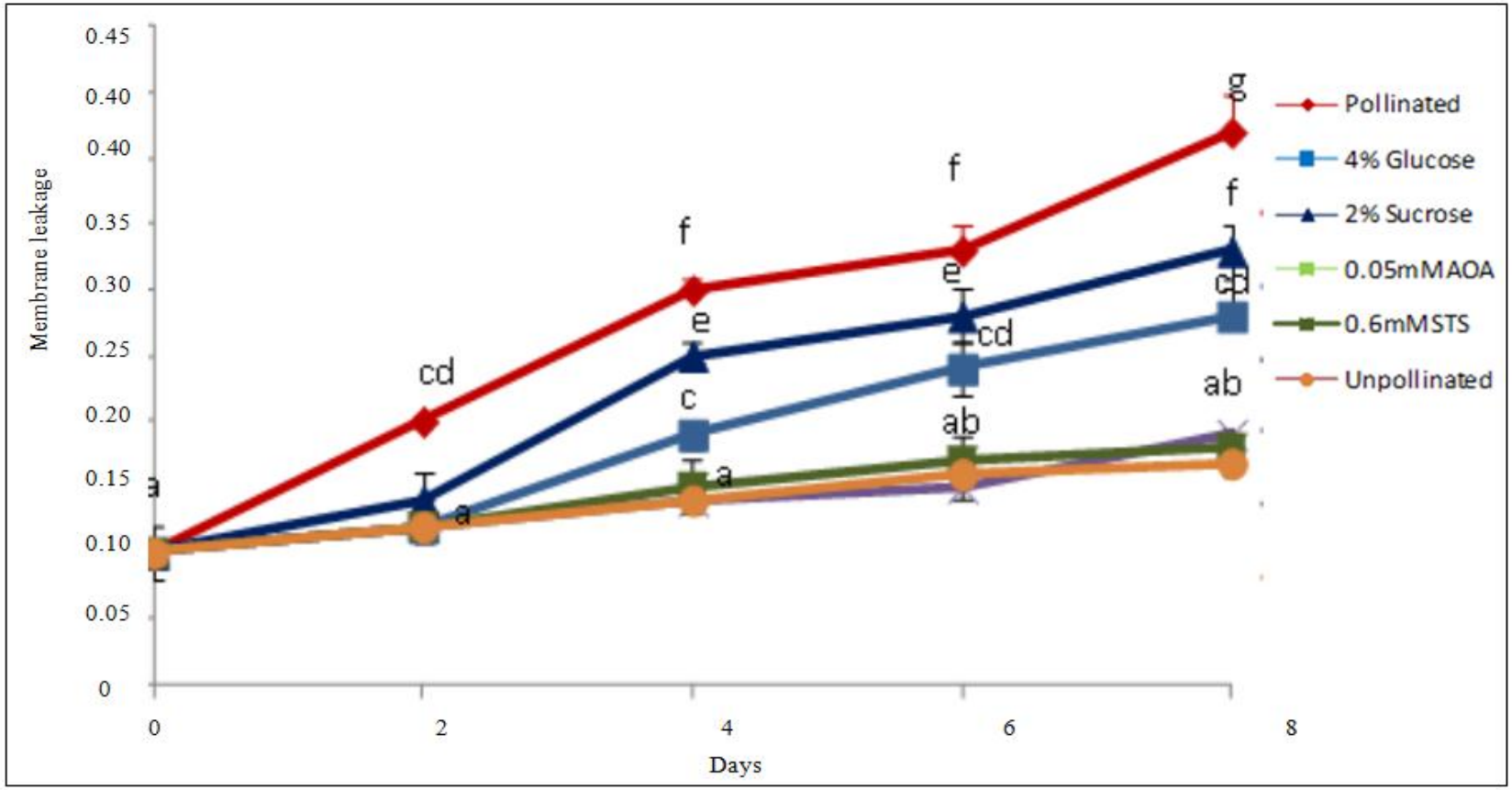


**Fig 5.5 Membrane leakage of unpollinated, pollinated and treated *D.* Pompadour flowers**. Each value is the mean ± SE ($n$ = 9), and values with different letter(s) are significantly different according to the Duncan's Multiple Range Test ($P < 0.05$).

## 5.4 DISCUSSION

Architectural modifications in cell wall which are being degraded are caused by the cumulative action of cell wall hydrolases. Amongst the various hydrolases, four were assayed in this study; polygalacturonase, pectatelyase, pectin methyl esterase and cellulase. The degradation of pectin occurs via a number of different mechanisms which include demethylesterification and solubilization. Pectin solubilization is usually measured as the increase in ease of hextractability of pectins by various extractants, and can be extrapolated to bond changes within the cell wall matrix (Brummell and Harpster, 2001).

Polygalacturonases (PGs) are cell wall-based enzymes that catalyze the hydrolytic cleavage of galacturonide linkages in pectins (Fischer and Bennett, 1991; Brummell and Harpster 2001). PG enzymes act mainly on the highly methylesterified form of homogalacturonans which are secreted into the plant cell wall (Carpita and Gibeaut, 1993; Minic and Jouanin, 2006). In tomato, a PG gene was isolated and was found to be involved in the regulation of pedicel abscission zone which suggests the likely involvement of PG in cell separation of flowers (Kalaitzis *et al.*, 1997). Using transgenic methods, PG was shown to be a major factor in regulation of pectin solubilization in fruits (DellaPenna *et al.*, 1987; Seymour *et al.*, 1987; Knapp *et al.*, 1989).Unlike PME, where no significant change in activity was observed, PG activity in pollinated *D.* Pompadour began to increase 2 days after pollination.

The post pollination increase in PG activity continued thereafter and peaked on the 6$^{th}$ day. This coincided with the deteriorated appearance of the flower where the flower is thin, shrivelled and appears necrotic. Rapid PG activity has also been reported in germinating and growing pollen tubes following pollination. High levels of exogenous PG have been reported in maize) and a number of trees (Pressey and Reger, 1989). Expression studies have revealed that an abundance of PG mRNA is present in mature pollen. This may also be the source of increased PG activity in pollinated flowers, because this enzyme is needed to degrade the walls of stylar cells to allow penetration of the pollen tubes (Mascarenhas, 1990; Pressey, 1991; Dubald *et al.,* 1993).

In daylily flowers, which begin to senesce 24 hours after they open, PG was undetectable during flower bud development until flowers fully opened. Initial increase started to show 12 hours after flowers opened and reached maximum activity 36 hours after that, a time at which the flowers had reached an advanced state of senescence. Anatomical changes showed that the peak PG activity in daylily coincided with the disintegration of cells and the disappearances of spaces between them (Panavas *et al.,* 1998).

In some flowers, PG activity was found to be insignificant throughout flower development. PG was undetectable all through the development in *D. Crumenatum* (Yap *et al.,* 2008), and continued to the senescence stage. In *Sandersonia* and carnations, very low PG activity was observed throughout the life of the flower

(O'Donoghue *et al.,* 2002; de Vetten and Huber, 1990).Whether a species specific variation occurs in terms of cell modification via PG is quite difficult to conclude as not enough data on the role of PG during flower senescence are available in the literature. Most studies on PG deal almost exclusively with cell wall modifications and softening of fruits. However, it is important to note that PG is efficient enough to catalyse pectin disassembly even at very low levels. This was evident in transgenic antisense tomatoes, where 80% reduction in PG activity did not slow down the disassociation of pectin structure (Smith *et al.,* 1990). It is therefore possible that PG dependency may still exist in species with low PG activity.

Pectate lyases (PL) facilitate cell wall disassembly by their action on de-methylated pectins through beta elimination in the presence of $Ca^{2+}$ leading to depolymerization of pectic polysaccharides (Kikuchi *et al.,* 2006). The activity of PL was found to undergo changes in pollinated *D.* Pompadour. However, the rate of activity of this enzyme was lower compared to that of PG activity. In the process of abscission, the dissolution of the middle lamella is of major importance because it is the structure that binds adjacent cells together. PL, along with the other pectin related hydrolases have been reported to act on pectin thereby facilitating progression of cell separation (Patterson, 2001; Jarvis *et al.,* 2003; Singh et al., 2011).

A study on the transcription of cell wall modification in *Arabidopsis* revealed 26 pectate lyase genes in flowers, most of which were expressed in high levels.

No conclusive evidence was obtained to relate pectate lyase to the senescence or abscission of *Arabidopsis.* However it was suggested that the genes could be involved in cell wall rearrangements during petal expansion (Palusa *et al.,* 2007). Similar restructuring events have been linked to pectate lyase in *Zinnia* (Domingo *et al.,* 1998). PL, just as is the case of PG was also reported to be involved in post pollination developments, which include growth of the pollen tube. Wing *et al.,* (1989) reported expression of PL in pollen and growing pollen tubes of tomato anthers. It was postulated that this enzyme brought about the release of oligogalacturonide fragments that have been found to serve as elicitors of ethylene biosynthesis (Felix *et al.,* 1991). A continuous increase in the transcription of PL in growing pollen tubes have also led to the proposition that the interaction between cell wall hydrolases and mechanical wounding of the stylar canal due to the growing pollen tubes sustain ethylene biosynthesis (Hoekstra and Weges, 1986).

PME catalyzes the removal of methyls from pectins. Grignon and Sentenac (1991) suggested that PME activity causes loosening of weakly attached pectins from the cell wall, thereby intensifying pectin solubilisation. It has been also suggested that PME prepares the cell wall for hydrolysis by polygalactouronase, which attacks demethylated residues (Carpita and Gibeaut, 1993). PME activity in *D.* Pompadour was low with little change detected from fresh, open flowers to senesced ones. Similar observations were also reported in senesced pollinated *D.* Savin White and *D.* Sonia

flowers (Ketsa *et al.,* 2000). In tobacco flower abscission zones, no changes were found in PME activity (Moline *et al.,* 1972) although PG activity increased. PME activity was similarly low in *Sandersonia* flowers (O' Donoghue, 2002), but a significant decrease was still observed in wilted and senesced flowers compared to mature flowers. In *Dendrobium crumenatum,* maximum pectin solubilisation was reported to coincide with peak PME activity during at the earliest stage of bud development. A significant decrease in PME activity was exhibited thereafter and PME activity remained constantly low until senescence. One would expect a parallel decrease in pectin solubility, however, this was not the case as pectin solubilisation continued to increase. This may be due to continuous solubilisation by other pectin hydrolases (Yap *et al.,* 2008).

In daylily, PME activity decreased from the young floral buds stage to flower opening, and subsequently increased during senescence (Panavas *et al.,* 1998). O'Donoghue (2002) suggested that these variations may be a consequence of differences in longevity and time frames of floral development from bud to senescence. Due to these variations, the exact role of PME during senescence cannot be ascertained. Furthermore, since cell wall modification occurs at many stages of flower development and is altered by a variety of cell wall hydrolases, it is reasonable to suggest that either PME does not play an important role in pectin degradation during pollination induced senescence in *D.* Pompadour, or is unable to function. A study on *Arabidopsis* reported that PME isoforms are coded by a multigene family with 67 PME related genes (Kaul *et al.,* 2000).

This may also explain the inconsistencies which were observed between enzymatic activity of PME and gene transcription in *D. Crumenatum.* The enzyme activity profile may be the product of two or more highly homologous genes (Yap *et al.*, 2008).

Modifications in cell wall glucans occur due to the actions of endo-1,4-β-glucanase or more commonly referred to as cellulase (Wood and Bhat 1988; Brummell and Harpster 2001). Cellulase catalyses the hydrolysis of linkages of 1,4-β-D-glucan chains adjacent to unsubstituted residues (Brummell and Harpster 2001). In pollinated *D.* Pompadour, cellulase was found to increase continuously throughout the experiment. A significant increase was observed in unpollinated flowers on day 8Ithas been suggested that cellulase activity may be involved in cell wall break down allowing sugars to be mobilized to other parts of the plants (Panavas and Rubeinsten, 1998). This observation supports earlier studies that confirmed the conversion of dayliliy petals from sink to source organs 12 hours after opening (Panavas *et al.*, 1998). This could explain the pattern of cellulase activity in pollinated *D.* Pompadour in this study. After pollination, the perianth becomes a source organ which provides energy and nutrients to developing embryos in the ovary. The activity of cellulase together with other cell wall hydrolases may be a feature of flower development with an ultimate result being pronounced changes in flower anatomy and nutrient remobilization.

In *D. crumenatum,* the synthesis of cellulose, the principal constituent of cell walls, was observed to increase concomitantly with the increase in cell wall materials during floral bud development. On the other hand, extensive breakdown of cellulose and hemicellulose were observed following pollination. Such massive modifications may be the cause for the total disintegration of the cell walls and the collapse of the parenchyma layers upon senescence of the flower (Yap *et al.,* 2008). Similar observations have also been reported in cut carnations where senescing flowers exhibited extensive loss in large size hemicelluloses polymers (de Vetten *et al.,* 1991). Interestingly, specific activity of cellulase was found to decrease considerably in the gynaecium of daylilies almost immediately after flower opening. This could be related to the development of the gynaecium tissue and its role in reproduction (Konar and Stanley, 1969; Panavas *et al.,* 1998).

Although many reports have shown that increases in cellulase activity are more often than not in sync with senescence or its onset, in some flowers cellulase activity begins much earlier. In Japanese morning glory for instance, insoluble solids concentrations increased during flower expansion and decreased thereafter, largely as a result of hemicelluloses and cellulose loss. Cellulase activity was found to significantly increase from the moment the flower opened and continued to increase to the point of flower senescence. (Wiemken-Gehrig *et al.,*1974).

The basic structure of the plant membrane consists of a phospholipid bilayer, with lipids, carbohydrates and proteins as its constituents. Membranes act as barriers that enclose plant cells. The integrity of membranes allows cells to perform multiple processes and functions which include import and export of nutrients and substances and the separation of enzymes from substrates. Loss in differential permeability of the cell membrane is a constant feature in plant senescence (Thompson, 1988, Paliyath and Dolliard, 1992). In both ethylene sensitive and insensitive flowers, considerable changes occur in the biochemical and biophysical properties of the membranes as a result of losses in phospholipids, increases in sugars, sterol to phospholipids ratio, and saturation to unsaturation index of fatty acids (Lesham, 1992; Thompson *et al.,* 1998).

Membrane senescence is evident visually as wilting and thinning of petals and internal cellular changes which include leakage of components such as enzymes, free radicals and electrolytes (Suttle and Kende, 1980). In flowers like roses and *Ipomoea*, phospholipid decline preceded visual senescence (Borochov *et al.,* 1997; Ferguson and Simon, 1979). The production of ethylene has been implicated in the hastening of senescence and the physiological and biochemical processes that it entails. Ethylene acts as a signal that orchestrates modifications in cell wall and membrane organization that eventually results in total loss of membrane integrity.

In the previous chapter, climacteric rise of ethylene in *D.* Pompadour post pollination was described and was found to be in concert with changes in perianth structure which includes loss of fresh weight and thinning of petals. In carnations, disruption of membrane was found to coincide with the climacteric rise of ethylene. Similar observations were found in *Ipomoea and Tradescantia* flowers where in the former, phospholipids disruption was elevated after ethylene synthesis whereas in the latter, climacteric rise of ethylene increased phospholipids catabolism which resulted in 70% phospholipid loss (Suttle and Kende, 1980).

Electrolyte leakage is indicative of the loss of membrane permeability and is a method commonly used to gauge the extent of loss in semi permeability and membrane integrity. Lypoxygenase (LOX), an enzyme which oxidizes phospholipids and galactolipids has been implicated in the loss of membrane function. In daylily, carnations and roses increased LOX activity was observed prior to detectable electrolyte leakage (Fobel *et al.*, 1987; Panavas and Rubenstein, 1998; Fukuchi *et al.*, 2000). Hong *et al.*, (2000) showed that an ethylene-inducible lipase mRNA increases just as carnation flowers begin to senesce. In *Alstromeria* flowers, while little electrolyte change was detected at the initial stages of inrolling of petals, electrolytes increased significantly at the advanced stages of senescence. In contrast, relative conductivity was significantly increased in pollinated *D.* Pompadour. By day 8, electrolyte leakage increased to 50%, concomitant with the further deterioration of the perianth structure.

In pollinated petunia flowers changes in lipid content and disorganization of membrane structure were observed before electrolyte leakage was detectable. The disruption of membrane integrity results in the decompartmentalisation of the cell and undesirable contact between cellular substances. This results in increased cellular disruption which in turn elevates cellular permeability and leakage of electrolytes (Marangoni *et al.,* 1996).

Treatments with sugars and ethylene inhibitors have extended vase life of pollinated *D.* Pompadour blooms and retained the quality of flowers. Results described in the previous chapter, showed that these treatments not only delayed closure of the flower, but maintained fresh weight, petal thickness, and dry matter content and resulted in an overall better flower appearance compared to controls. These symptoms also reflect the maintenance of cell wall structure and membrane integrity. Treatments described in this chapter delayed the increase in PG, PL and cellulase activity in pollinated flowers.

The effectiveness of sugars, such as sucrose in maintaining flower quality can be attributed to its role as a substrate which is used as an energy by the flower. In the progression of senescence where energy sources in the form of respirable substrates decrease in concentration, flowers utilize alternative sources, one of which is the cell wall. (van Doorn, 2004). As was mentioned earlier, cell wall disruption during flower senescence is a form of nutrient remobilization.

In sucrose fed *Sandersonia* petals, reduction in the quantity of soluble pectins was observed. It was reported that sucrose may have made available residues for polymer assembly and facilitated in the activation of wall synthesis enzymes (O'Donoghue *et al.*, 2005). In carnations, non-treated flowers showed higher levels of highly charged, branched and more soluble pectins whereas sucrose-fed flowers showed reduction in pectin (de Vetten and Huber, 1990). Work with *Arabidopsis thaliana* showed that an interaction exists between glucose, sucrose and ethylene. It was suggested that sucrose may temporarily impair the activity of ACCO and ACCS as exhibited by their low activity in sucrose treated pre-senescent carnations exposed to ethylene (Verlinden and Garcia, 2004). This was further confirmed at the transcriptional level, when the application of sugars was found to delay the accumulation and EIN3-like MRNA in carnations (Iordechescu and Verlinden, 2005). Considering the interaction between sugars and ethylene, it is of no surprise that sucrose treatments are less effective in delaying senescence in ethylene insensitive flowers.

The addition of ethylene inhibitors was very effective in inhibiting cell wall hydrolases and membrane integrity of flowers. Blocking of ethylene production and sensitivity to it eliminates the signal that brings about cell wall and membrane modification during pollination induced senescence. Pre treatment of petiole explants of *Coleus* blumei with STS prevented ethylene-induced reorganization of the endogenous membrane system and the subsequent middle lamellar dissolution (Baird *et al.*, 1984). The application of ethylene inhibitors and their effects on cell wall modification have investigated extensively in fruit ripening and softening.

The use of AOA in oranges resulted in an absence of pectin solubilisation for 72 hours when on the contrary the controls softened rapidly. In Ambersweet oranges where rapid PG and cellulase activity was detected upon ethylene exposure, a 22% and 35% reduction occurred respectively when AOA was applied. The effect of STS on cell wall hydrolases have also been reported at a transcriptional level with the ability of suppressing accumulation of abscission specific PG enzymes in tomatoes (Kalaitzis *et al.*, 1995).

The findings obtained in this chapter show the involvement of cell wall hydrolases and the hastening of their activity during pollination-induced senescence in *D.* Pompadour. Furthermore, membrane integrity was also compromised in pollinated flowers. Treatments using sugars and ethylene inhibitors have successfully delayed and reduced the rate of cell wall hydrolase activity and membrane leakage.