CHAPTER 6
PROTEIN CONTENT AND PROFILE ANALYSIS OF POLLINATED FLOWERS HELD IN DIFFERENT TREATMENT SOLUTIONS

6.1 INTRODUCTION

Protein loss is a common event during flower senescence and has been reported in many flowers. Large losses in the protein content prior to senescence have been observed in flowers such as *Ipomoea, Hemerocallis* and *Petunia* (Matile and Winkenbach, 1971; Lay-yee *et al.,* 1992; Gulzar, 2003). Pollination-induced senescence has also been associated with protein loss as is the case with *D.* Khao Sanan (Lerslerwong *et al.,* 2009) *Cymbidium* (Attri *et al.,* 2008) and *petunia* (Xu and Hanson, 2000). Decrease in protein abundance is accompanied by increase in proteolytic activity and amino acids. Like other complex molecules, proteins are broken down to supply nitrogen in the form of amino acids to developing tissues (Soudry *et al.,* 2005).

The change in protein status involves both soluble and insoluble proteins. Soluble proteins are mainly made up of enzymes while insoluble proteins are predominantly made up of wall membrane proteins. In a number of flowers, pollination was found to have a greater effect on the decrease of insoluble proteins compared to the soluble ones (Lerslerwong *et al.,* 2009). Nevertheless, this is not a universal observation as observed in the significant decrease of soluble proteins in other flowers (Eason *et al.,* 2002; Wagstaff *et al.,* 2002; Jones *et al.,* 2005).

As a whole, the status of total protein during pollination induced senescence is a combination of both degradation and synthesis of proteins. For example, inhibiting *de novo* synthesis of proteins in carnation (Wulster *et al*., 1982), daylily (Lukaszewski and Reid, 1989; Lay-Yee *et al*., 1992), *Gladiolus* and *Narcissus* (Jones *et al*., 1994) successfully delayed senescence. In *Sandersonia,* inhibiting protein degradation via cysteine protease inhibitors managed to delay tepal wilting (Eason *et al.,* 2002).

As a highly controlled developmental process, it could be assumed that pollination induced- senescence, must be regulated at the level of transcription and translation. The synthesis of new proteins may be necessary to regulate this process (Woodson and Handa, 1987). Specific proteins have been reported to be either up or down regulated in a number of flowers undergoing senescence. A 39 kDa and a 61 kDa protein, for instance were found to be up regulated in senescing carnations and coincided with the peak of ethylene production (Burchi *et al.,* 1993). Although no further analyses were carried out to specifically identify those proteins, they were speculated to be similar proteins that responded to abiotic stress in plants (Burchi *et al.,* 1993). Proteomic analysis on pollinated *petunias* revealed that a majority of upregulated proteins were involved in defence pathway and macromolecule catabolism (Bai *et al.,* 2010).

N-terminal sequencing performed by Edman degradation is a reliable technique for obtaining N-terminal sequence of intact proteins. This automated method involves a series of chemical reactions that remove one amino acid at a time from the N-terminus of purified peptides or intact proteins. With a sensitivity of 5-10 pmol, 7-10 protein residues are sufficient for protein identification. These features make it a suitable method for analysing proteins that have been separated by 1D SDS PAGE and subsequently transferred to a PVDF membrane.

The aim of this chapter is to investigate the changes that occur in protein content and the profile of *D.* Pompadour flowers following pollination as well as flowers held in treatment solutions. One Dimensional Sodium Dodecyl Sulphate Polyacrylamide (SDS) will be employed to separate proteins and observe the changes in the protein profiles of the flowers. Once the profile is obtained, some key polypeptides will be selected for further analysis of N-terminal sequencing performed by Automated Edman degradation. The use of bioinformatic tools will assist in protein homology analysis and link the proteins to their specific function.

6.2 MATERIALS AND METHODS

6.2.1 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.

6.2.2 Extraction of Total Soluble and Insoluble Proteins

Five grams (fresh weight) of flowers held in distilled water and different treatment solutions were ground to fine powder in the presence of liquid nitrogen using a mortar and pestle. Soluble proteins were extracted by adding 15 ml of extraction buffer (Chandran, 2004). The mixture was then vortexed thoroughly and incubated at 25^{o}C for 30 minutes. Following incubation, the mixture was spun at 14,500 rpm, for 20 minutes at 4^{o}C using a Sorvall RC5C refrigerated centrifuge. Upon centrifugation, the supernatant was collected by filtering through one layer of Mira cloth while the pellet was further used for insoluble protein extraction. Insoluble proteins were extracted by resuspending the pellets in 10 ml of 0.1 M NaOH and incubating the mixture at 80^{o}C overnight. The protein content was estimated using the Bradford (1976) method with Bovine Serum Albumin as a standard. Total protein was estimated as a sum of both soluble and insoluble proteins.

6.2.3 One Dimensional (1-D) SDS PAGE Analysis

Total soluble proteins in the flower extract were separated by 10% SDS PAGE gels according to the Laemmli method (1970) using a Mini Protean II System (Bio-Rad). 30µg of protein was applied to each lane and detection was carried out using the Coomasie Blue staining. Pre-stained protein molecular markers were included for molecular size determinations. Gels were run in 1L of tank buffer (Tris-Glycine) at an initial current of 60 mA and a constant voltage of 100 V. After electrophoresis, the gel was carefully removed and quickly rinsed in dH_2O and stained with Coomasie Blue R250 for six hours to visualize polypeptide bands. Destaining was done using a buffer containing 40% (v/v) methanol and 10% (v/v) acetic acid until the bands were visible against the background.

6.2.4 Polyvinylidenedifluoride (PVDF) Blot Transfer of Polypeptides

For protein sequencing and amino acid analysis the proteins were transferred to a chemically stable membrane, PVDF. After proteins were separated by SDS-PAGE, the gel was removed from the electrophoresis cassette and equilibrated for five minutes in 100 ml of transfer buffer consisting of 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) buffer without methanol. The PVDF membrane was "activated" by dipping it in methanol; then placed in transfer buffer containing methanol. The transblot sandwich was assembled in the following order starting from the anode side: sponge, filter paper, 2 sheets of PVDF membranes, gel, filter paper, and sponge. The gel-PVDF sandwich was placed in a specially designed holder that in turn was placed in the buffer-containing electrophoresis unit. Electroblot was carried out at room temperature at a constant voltage (50V) for 30 minutes. At the pH of the buffer (pH 8.3) most proteins are negatively charged and will migrate to the anode (positive electrode). After completion of the electroblot, the PVDF membranes were removed and

rinsed in sterile distilled water and saturated with 100% methanol for a few seconds. Protein samples were detected with Coomasie blue. Each PVDF membrane was stained one at a time using constant orbital shaking for about one to three minutes (until the bands were visible) and then was destained with 50% methanol. The membranes were then rinsed in sterile distilled water, dried and the polypeptides of interest were marked on the side of the blot with a pencil.

The N-terminal sequences of specific polypeptides were determined by automated Edman degradation using an ABI Procise 494 Protein Sequencer in the Protein and Proteomics Centre, PPC S3-03, Department of Biological Sciences, National University of Singapore.

6.2.5 Bioinformatic Analysis Tool

BlAST NCBI was accessed via the NCBI (National Center for Bioinformatics USA) website; www.ncbi.com. The programme is widely used for searching DNA and protein databases for sequence similarities. Protein blast or blastp is used in this study to search a number of non- redundant protein databases for similarities with the query sequence.

6.3 RESULTS

6.3.1 Protein Content in Pollinated *D.* Pompadour Flowers

Figure 6.1 shows total protein content in *D.* Pompadour flowers. Total protein content of unpollinated flowers remained unchanged throughout the experiment. Similarly, flowers held in 0.05 mM AOA and 0.6 mM STS also did not exhibit significant change in total protein content. In contrast, a significant decrease was observed in pollinated flowers beginning 2 days after pollination. Total protein of flowers held in 4% glucose and 2% sucrose significantly decreased 6 days after pollination. By the end of the experiment, pollinated flowers and flowers held in 4% glucose and 2 % sucrose lost a total percentage of 47%, 29% and 35% of total protein respectively.

In all flowers except for the pollinated ones, soluble protein content increased on day 2 and decreased at varying rates at the end of the experiment (Fig 6.2). Unpollinated flowers and flowers held in 0.05 mM AOA showed insignificant change in soluble protein by the end of the observation. Pollinated flowers and flowers held in 4% glucose, 2% sucrose and 0.6 mM STS retained 75%, 88%, 82% and 92% respectively.

As shown in Figure 6.3, no significant change was observed in the amount of insoluble protein of unpollinated flowers, and flowers held in 0.05 mM AOA and 0.6 mM STS. Pollinated flowers showed rapid decrease of insoluble protein with only 20% protein left at the end of the experiment. Similar trend was observed in flowers held in 4% glucose and 2% sucrose with only 40% and 37% insoluble protein left at the end of the observation.

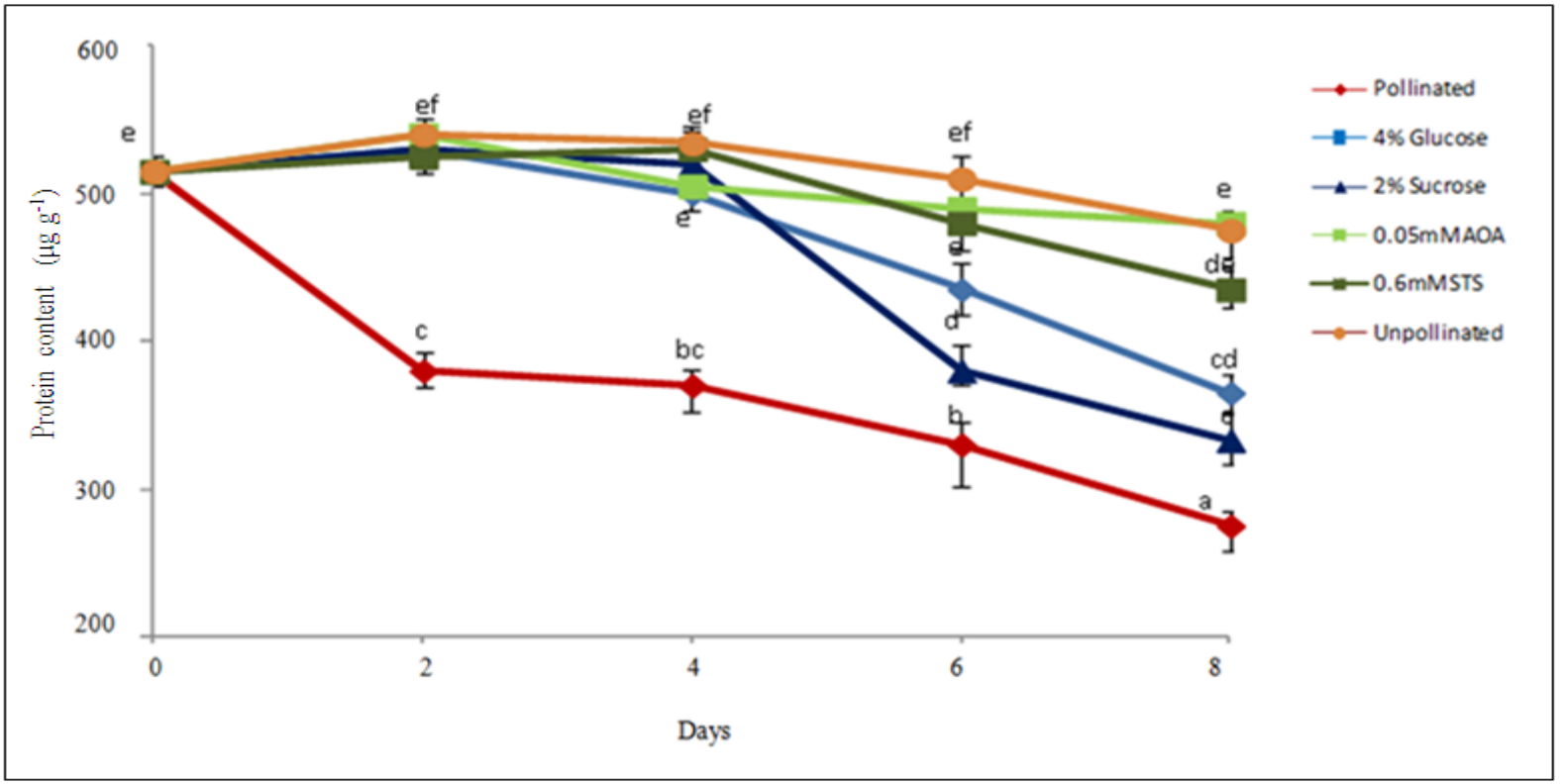

Figure 6.1: Total protein content in unpollinated, pollinated and treated *D.* Pompadour flowers. Each value is the mean ± SE (*n* = 9), and values with different letter(s) are significantly different according to the Duncan's Multiple Range Test (P < 0.05).

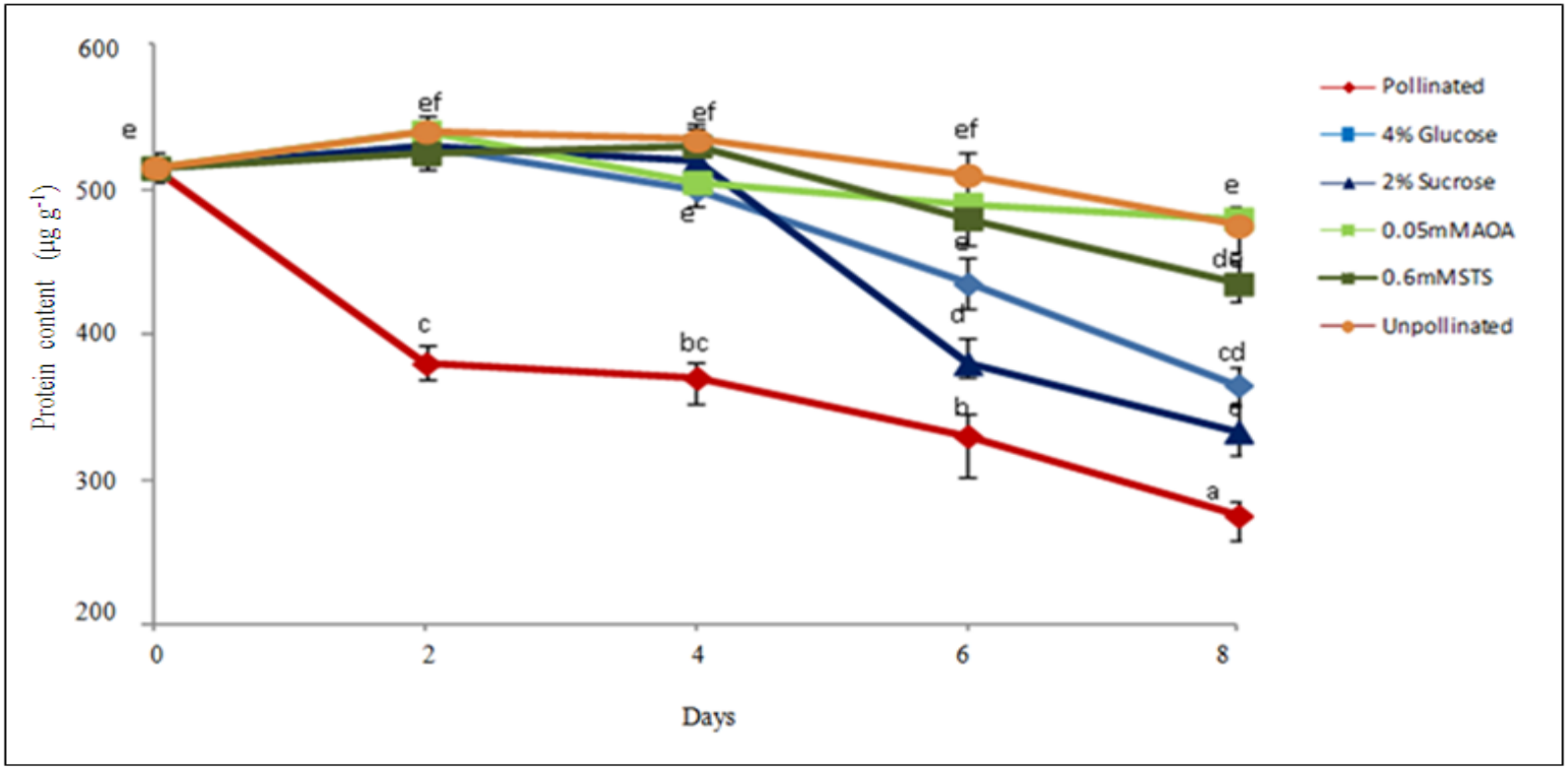

Figure 6.2: Total soluble protein content in unpollinated, pollinated and treated *D.* Pompadour flowers. Each value is the mean ± SE (*n* = 9), and values with different letter(s) are significantly different according to the Duncan's Multiple Range Test (P < 0.05).

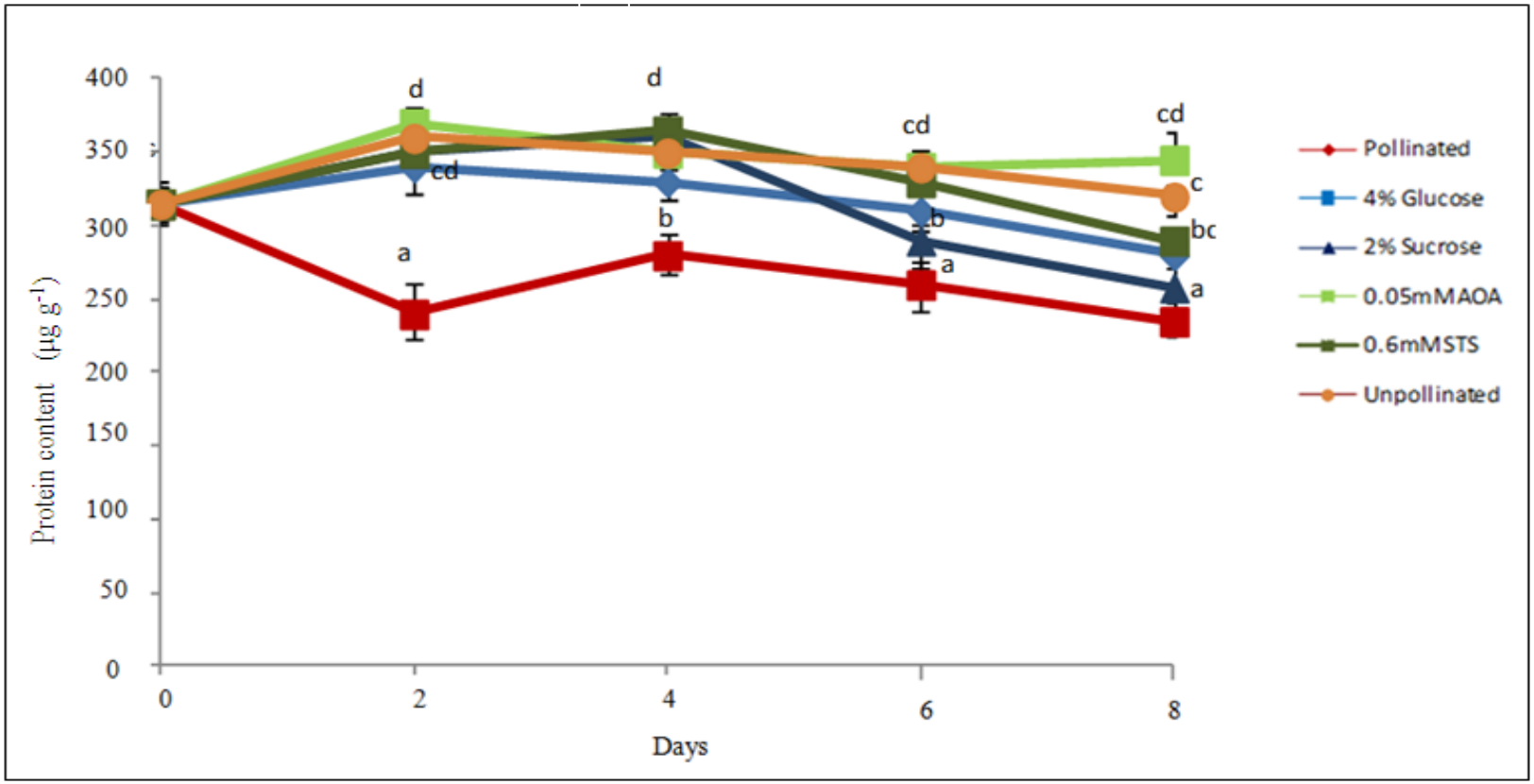

Figure 6.3: Total insoluble protein content in unpollinated, pollinated and treated *D.* Pompadour flowers. Each value is the mean ± SE (n = 9), and values with different letter(s) are significantly different according to the Duncan's Multiple Range Test ($P < 0.05$).

6.3.2 SDS PAGE Analysis of Protein Profile in Unpollinated, Pollinated and Treated *D.* Pompadour Flowers

The protein profile (10% SDS PAGE) for unpollinated *D.* Pompadour flowers is shown in Fig 6.4 (A). Three key polypeptides based on abundance and distribution were detected. Based on the Rf values and molecular weight markers the polypeptides were sized as 30 kDa, 27 kDa and 15 kDa. From the visual observation of the gel, both the polypeptides sized 30 and 27 kDa were found to be most abundant in unpollinated *D.* Pompadour. On the other hand, the 15 kDa polypeptide remained unchanged.

In order to confirm the abundance of these polypeptides, density values were calculated using the EZQuant Gel 2.2 Software. The generated gel picture is shown in Fig 6.4B. For the purpose of a relative comparison an arbitrary score of 1.0 was accorded to the polypeptides expressed at the beginning of the experiment (D0). The same polypeptides expressed on subsequent days were scored relative to these to give an indication of how abundant these polypeptides were distributed within a profile. The results are summarised in Table 6.1. Based on the scores, it was found that the 30 kDa was most abundant on day 2 with a 1.9 increase from that expressed on day 0. The 27 kDa polypeptide did not increase in abundance. The 15 kDa polypeptide remained constant throughout the experiment, except for a decrease on day 8. Overall it can be summarised that in unpollinated *D.* Pompadour, significant increase occurred in the expression of the 30 kDa protein, while no change was observed in both the 27 and 15 kD apolypeptides except for 15kDa polypeptide on day 8.

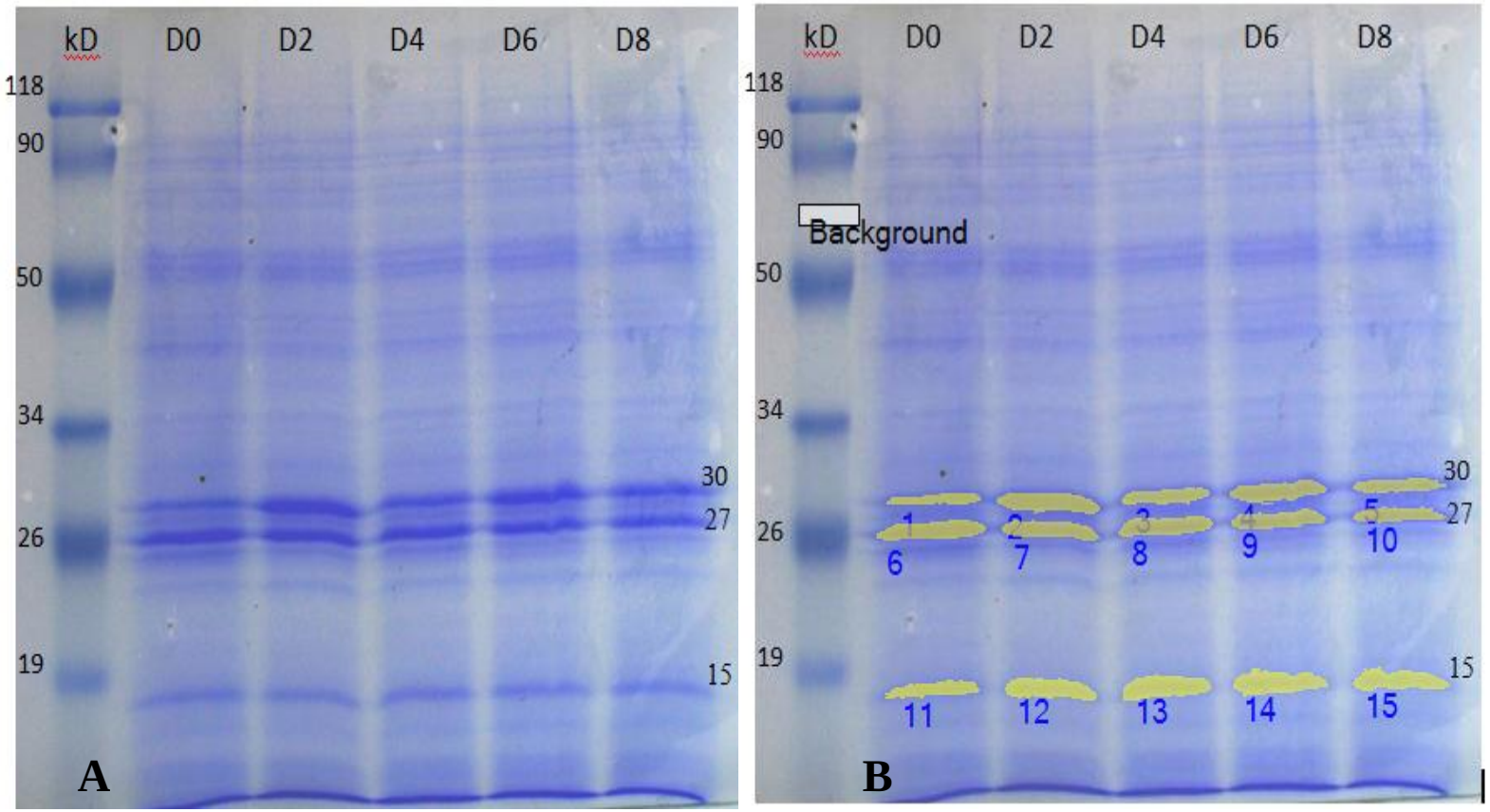

Fig 6.4 (A-B). A) Pattern of protein changes in unpollinated *D.* Pompadour. Representative gel from triplicates of total soluble protein from unpollinated flowers extracted every 2 days for 8 days. Proteins were separated by 10% SDS-PAGE and visualized after staining with Coomassie blue R. Each lane was loaded with 30 µg of total protein extract. Proteins with molecular masses of 15, 27 and 30 kDa were detected. Prestained Protein Molecular Weight Markers (Fermentas) are indicated on the far left. **B) Band detection image of pollinated flowers using the EZQuant 2.2 software.** The rectangle at the top left corner indicates the background against which the bands were measured.

Table 6.1 Relative abundance of polypeptides in unpollinated *D.* Pompadour flowers. The score is relative to the 1.0 assigned to the polypeptide expressed on day one (bands number 1, 6 and 11). The different colours indicate the different polypeptides.

Protein Size	Day	Band Number	EQuant Value	Score
30 kDa	0	1	121.0	1.0
	2	2	225.8	1.9
	4	3	152.5	1.3
	6	4	187.1	1.5
	8	5	139.9	1.2
27 kDa	0	6	231.0	1.0
	2	7	216.4	0.9
	4	8	216.3	0.9
	6	9	192.5	0.9
	8	10	151.3	0.8
15 kDa	0	11	113.7	1.0
	2	12	116.0	1.0
	4	13	117.0	1.0
	6	14	118.3	1.0
	8	15	120.2	1.1

After establishing the protein changes in unpollinated flowers, another profile was run to determine the changes of the three polypeptides in pollinated *D.* Pompadour flowers. Figure 6.5A shows the protein profile of pollinated flowers. Figure 6.5B shows the generated gel picture using EZQuant Gel 2.2 Software.

Based on the scores calculated (Table 6.2), The 30 kDa polypeptide was most abundant and increased from 1.0 to 2.6 and 2.7 on day 6 and day 8. The 27 kDa protein increased significantly on day 2 and decreased thereafter. This was contrary to that observed in unpollinated flowers where no increase was observed in the expression of this 27 kDa protein throughout the experiment. Finally, the 15 kDa generally increased throughout the experiment. Nevertheless, the abundance of this protein was less compared to the other two proteins. Overall, it can be summarised that significant changes were observed in all three polypeptides of pollinated flowers.

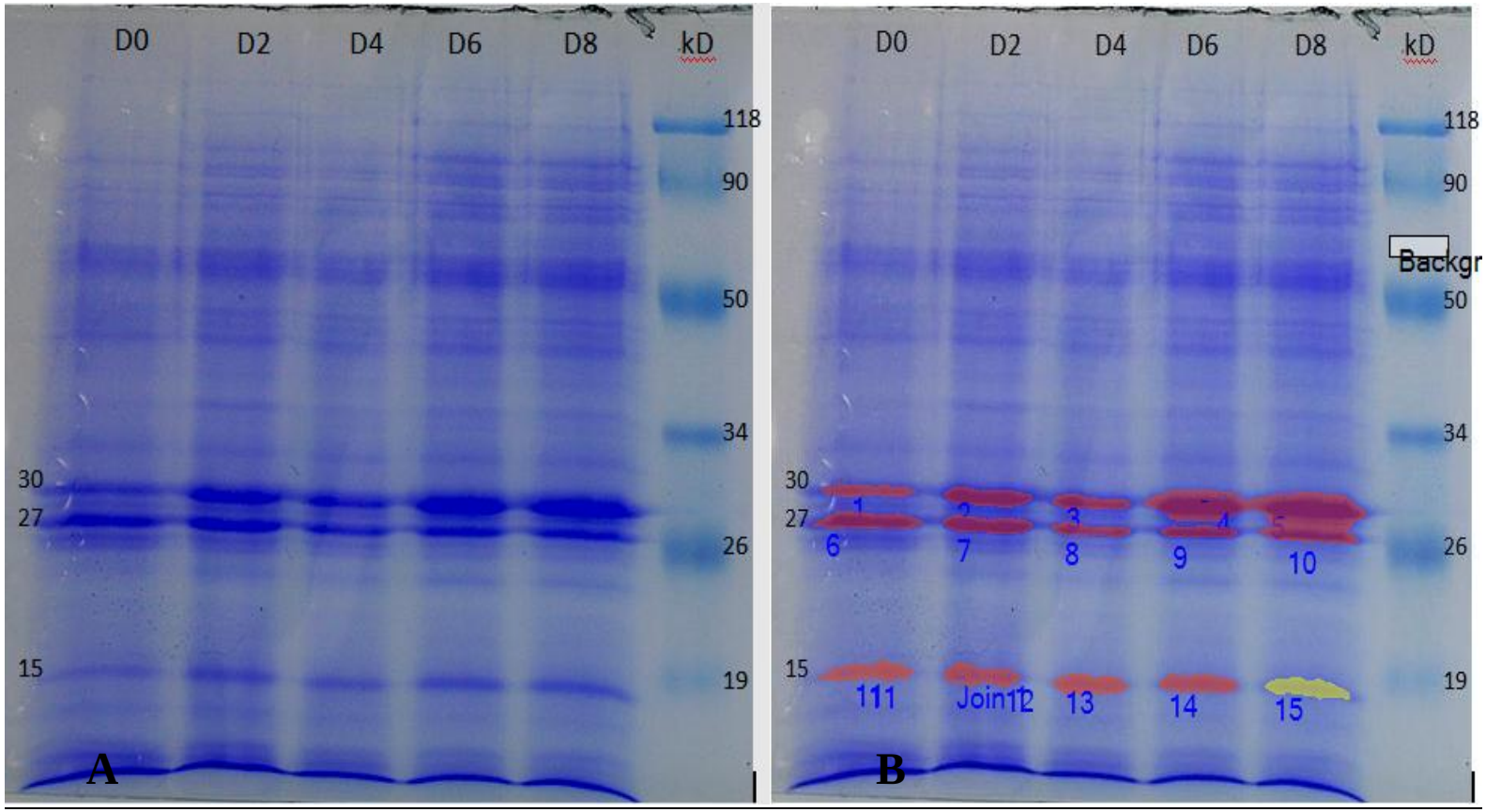

Fig 6.5 (A-B). A) Pattern of protein changes in pollinated *D.* Pompadour. Representative gel from triplicates of total soluble protein from pollinated flowers extracted every 2 days for 8 days. Proteins were separated by 10% SDS-PAGE and visualized after staining with Coomassie blue R. Each lane was loaded with 30 µg of total protein extract. Proteins with molecular masses of 15, 27 and 30 kDa were detected. Prestained Protein Molecular Weight Markers (Fermentas) are indicated on the far right. **B) Band detection image using the EZQuant 2.2 softwar**e. The rectangle at the top right corner indicates the background against which the bands were measured

Table 6.2 Relative abundance of polypeptides in pollinated *D.* Pompadour flowers. The score is relative to the 1.0 assigned to the polypeptide expressed on day one (bands number 1, 6 and 11). The different colours indicate the different polypeptides.

Protein Size	Days	Band Number	EZQuant Value	Ratio
30 kDa	0	1	239.6	1.0
	2	2	424.2	1.8
	4	3	257.4	1.1
	6	4	618.8	2.6
	8	5	645.5	2.7
27 kDa	0	6	440.5	1.0
	2	7	759.5	1.7
	4	8	611.9	1.2
	6	9	453.8	1.0
	8	10	489.1	1.1
15 kDa	0	11	177.6	1.0
	2	12	227.9	1.3
	4	13	216.8	1.2
	6	14	222.1	1.3
	8	15	210.1	1.2

The effects of different treatment solutions on the protein profiles of pollinated *D.* Pompadour flowers were also investigated. Fig 6.6A shows the protein profile of pollinated *D.* Pompadour held 4% glucose solution. The generated gel picture (EZQuant 2.2 Software) is shown in Fig 6.6B.

Based on Fig 6.6A, it was found that similar to unpollinated and pollinated flowers, the 30 and 27 kDa polypeptides was the most abundant. 15 kDa proteins were present albeit in lower amounts. It was also found that the 30 kDa protein was not detected on day 4.

Based on the scores (Table 6.3) it was observed that the 30 kDa protein was most abundant on day 2 and 4 increasing 1.6 folds. The 27 kDa polypeptide remained unchanged until day 4 before increasing significantly on day 6. A significant decrease in 15 kDa polypeptide was observed on day 2 (less than half of protein expressed on day 0) before increasing thereafter.

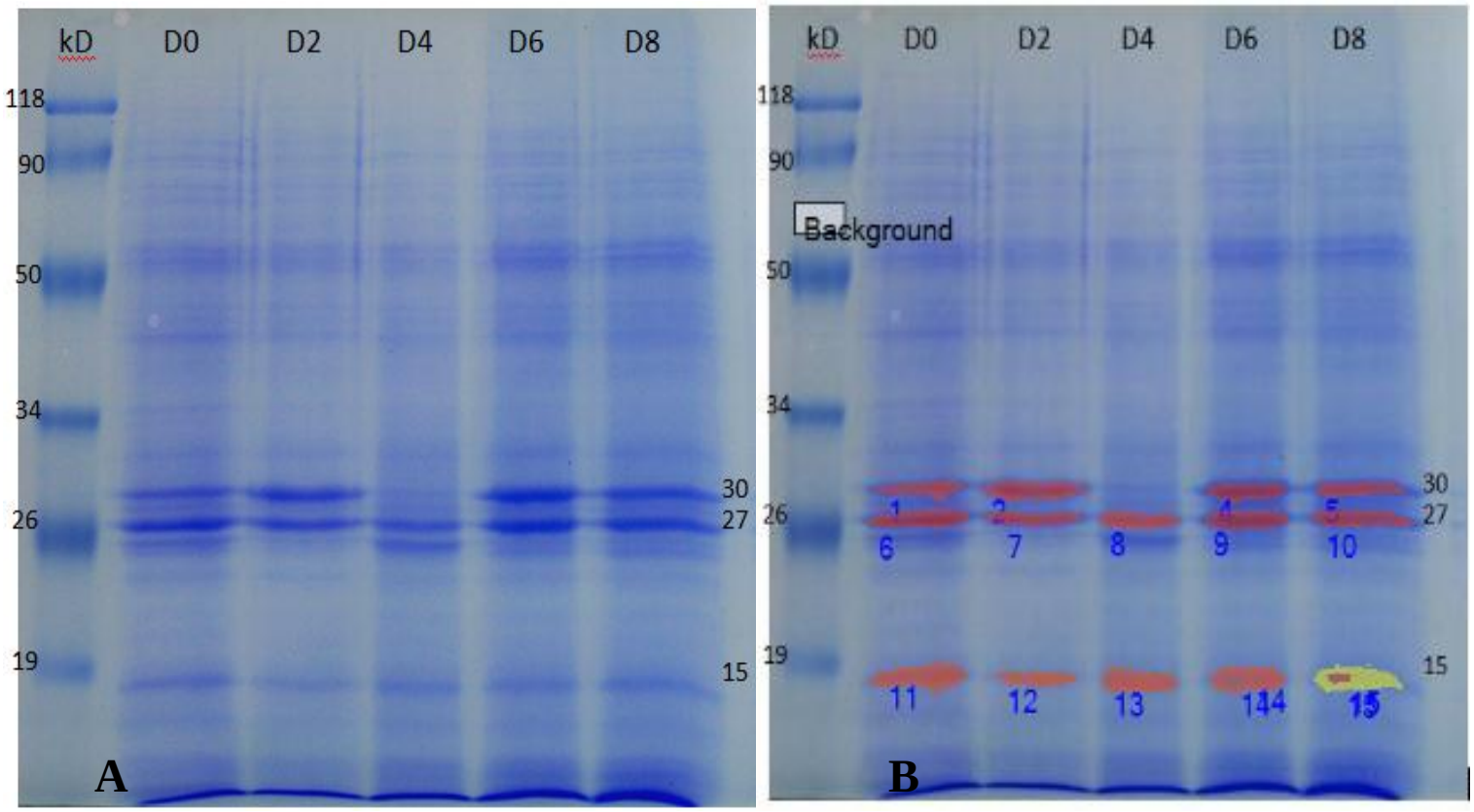

Fig 6.6 (A-B). A) Pattern of protein changes in pollinated *D.* Pompadour held in 4% glucose solution. Representative gel from triplicates of total soluble protein from flowers held in 4% glucose extracted every 2 days for 8 days. Proteins were separated by 10% SDS-PAGE and visualized after staining with Coomassie blue R. Each lane was loaded with 30 µg of total protein extract. Proteins with molecular masses of 15, 27 and 30 kDa were detected. Prestained Protein Molecular Weight Markers Fermentas) are indicated on the far right. **B) Band detection image using the EZQuant 2.2 software**. The rectangle at the top right corner indicates the background against which the bands were measured.

Table 6.3 Relative abundance of polypeptides in pollinated *D.* Pompadour flowers held in 4% glucose solution. The score is relative to the 1.0 assigned to the polypeptide expressed on day one (bands number 1, 6 and 11). The different colours indicate the different polypeptides.

Protein Size	Days	Band Number	EZQuant Value	Ratio
30 kDa	0	1	167.2	1.0
	2	2	271.5	1.6
	4	4	265.4	1.6
	6	5	210.4	1.3
27 kDa	0	6	300.1	1.0
	2	7	186.7	1.0
	4	8	177.7	1.0
	6	9	390.2	1.3
	8	10	329.9	1.1
15 kDa	0	11	199.1	1.0
	2	12	87.3	0.4
	4	13	153.2	0.8
	6	14	169.7	0.9
	8	15	180.3	0.9

Another profile was run to examine the changes of the sized polypeptides in pollinated flowers held in 2% sucrose solution (Fig 6.7A). Upon visual observation of the gel, the 30 kDa protein was found to increase in abundance on day 4 and day 6. On day 8, the polypeptide was present at trace amounts. The amount of the 27 kDa protein appeared to be consistent. As in flowers held in 4% glucose solution, the 15 kDa protein was also present in lower amounts.

The protein profile was further confirmed by the values obtained from EZQuant Gel 2.2. Software (Table 6.4). The scores were in agreement with visual observation as the 30 kDa protein recorded the highest score on day 4 with a 1.7 fold increase. The scores also showed that 27 kDa did not increase until day 6. Finally, the 15 kDa polypeptide was found to decrease until the end of the experiment, at this point the amount of protein was less than half compared to that on day 0.

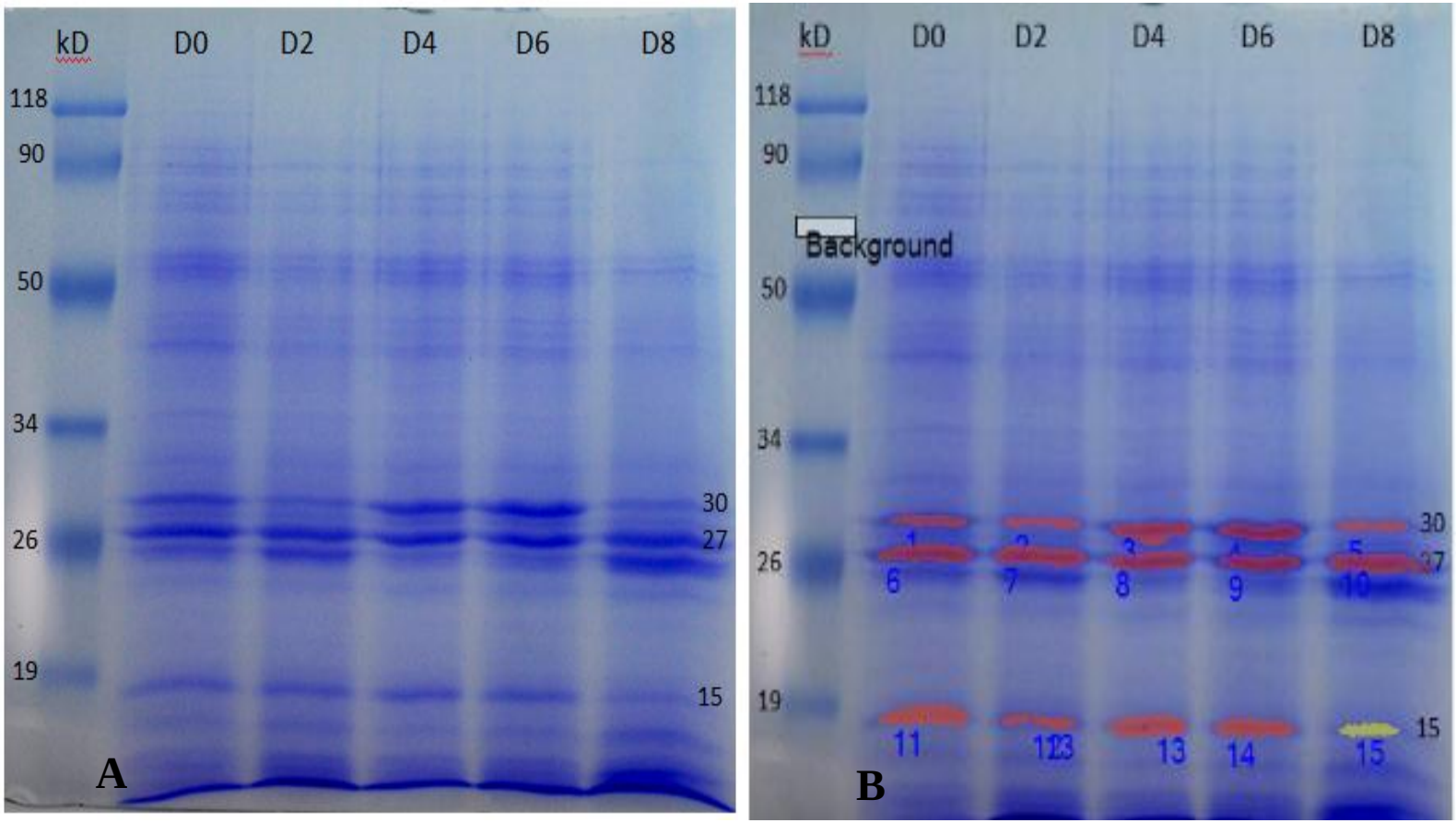

Fig 6.7 (A-B). A) Pattern of protein changes in pollinated *D.* Pompadour held in 2% sucrose solution. Representative gel from triplicates of total soluble protein from flowers held in 2% sucrose solution was extracted every 2 days for 8 days. Proteins were separated by 10% SDS-PAGE and visualized after staining with Coomassie blue R. Each lane was loaded with 30 µg of total protein extract. Proteins with molecular masses of 15, 27 and 30 kDa were detected. Prestained Protein Molecular Weight Markers (Fermentas) are indicated on the far right. **B) Band detection image using the EZQuant 2.2 software**. The rectangle at the top right corner indicates the background against which the bands were measured against.

Table 6.4 Relative abundance of polypeptides in *D.* Pompadour flowers held in 2% sucrose. The score is relative to the 1.0 assigned to the polypeptide expressed on day one (bands number 1, 6 and 11). The different colours indicate the different polypeptides.

Protein Size	Days	Band Number	EZQuant Value	Ratio
30 kDa	0	1	241.4	1.0
	2	2	142.3	0.6
	4	3	406.2	1.7
	6	4	318.2	1.3
	8	5	139.0	0.6
27 kDa	0	6	355.1	1.0
	2	7	370.7	1.0
	4	8	314.3	0.9
	6	9	433.1	1.2
	8	10	468.6	1.3
15 kDa	0	11	339.0	1.0
	2	12	318.7	0.9
	4	13	278.0	0.8
	6	14	247.5	0.7
	8	15	121.8	0.4

Protein changes in pollinated flowers held in 0.05 mM AOA were also established. Based on the gel (Fig 6.8A) a significant increase of the 30 kDa protein was observed on day 4 and 6. The 27 kDa polypeptide showed a more consistent profile except on day 2 where a decrease was observed. As in previous treatments, the 15 kDa polypeptide was also present in lower amounts.

The observations were confirmed objectively as shown in Table 6.5. Based on the scores, the 30 kDa was most abundant on day 6. The 27 kDa polypeptide significantly decreased on day 2, half of the amount expressed on day 0. Thereafter, the protein increased. Finally, on day 6 the amount of 15 kDa protein was half of what was expressed in the beginning of the experiment. After that, the protein increased.

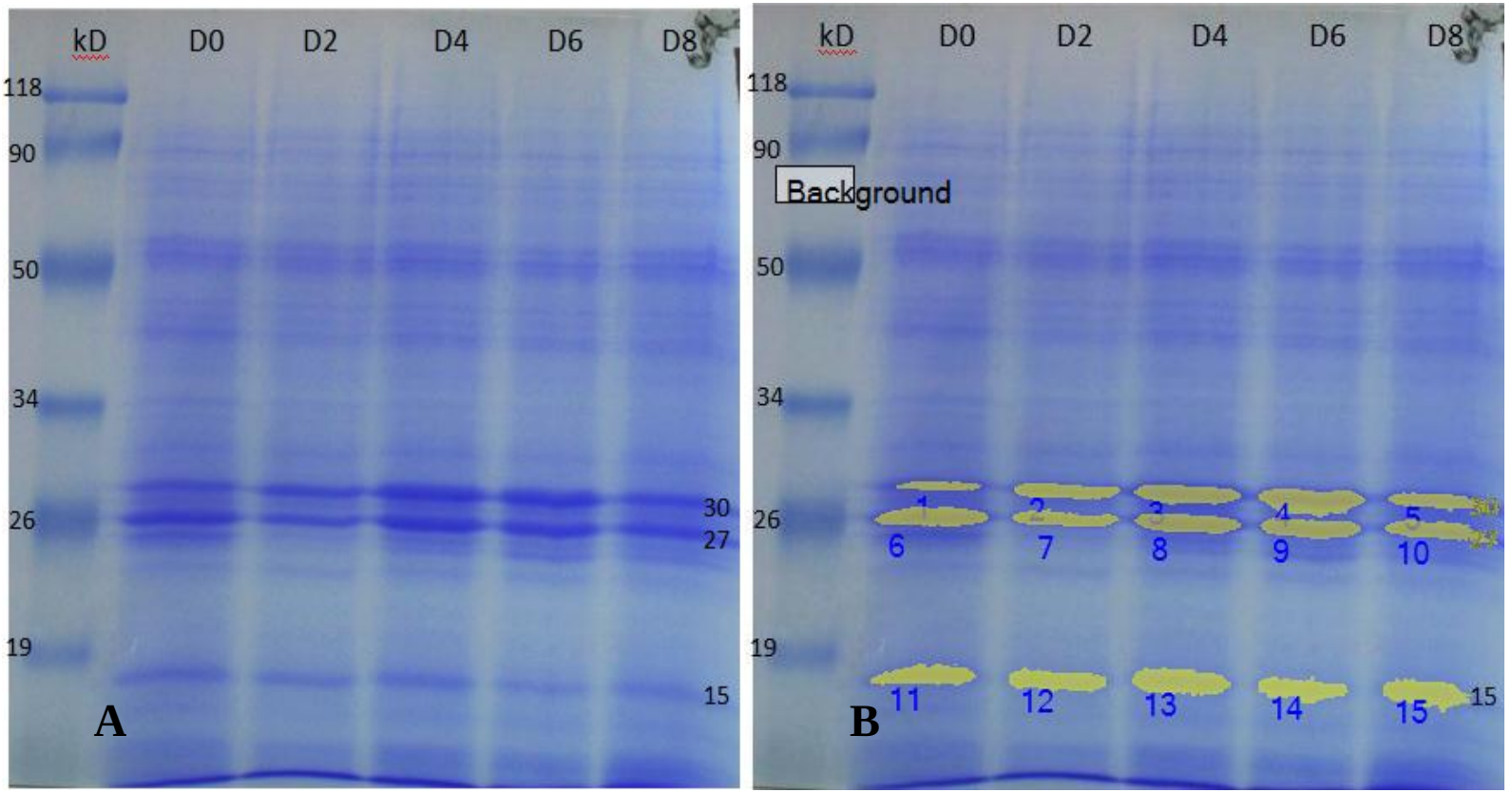

Fig 6.8 (A-B). A) Pattern of protein changes in pollinated *D.* Pompadour held in 0.05 mM AOA solution. Representative gel from triplicates of total soluble protein from flowers held in 0.05mM AOA solution extracted every 2 days for 8 days. Proteins were separated by 10% SDS-PAGE and visualized after staining with Coomassie blue R. Each lane was loaded with 30 µg of total protein extract. Proteins with molecular masses of 15, 27 and 30 kDa were detected. Prestained Protein Molecular Weight Markers (Fermentas) are indicated on the far right. **B) Band detection image using the EZQuant 2.2 software**. The rectangle at the top right corner indicates the background against which the bands were measured.

Table 6.5 Relative abundance of polypeptides in *D.* Pompadour flowers held in 0.05 mM AOA. The score is relative to the 1.0 assigned to the polypeptide expressed on day one (bands number 1, 6 and 11).The different colours indicate the different polypeptides.

Protein Size	Days	Band Number	EZQuant Value	Ratio
30 kDa	0	1	214.0	1.0
	2	2	238.0	1.1
	4	3	289.9	1.4
	6	4	348.9	1.6
	8	5	234.3	1.1
27 kDa	0	6	306.9	1.0
	2	7	175.6	0.5
	4	8	287.8	0.9
	6	9	252.3	0.8
	8	10	271.6	0.9
15 kDa	0	11	182.3	1.0
	2	12	138.0	0.8
	4	13	185.2	1.0
	6	14	100.7	0.5
	8	15	147.0	0.8

The final profile was run to establish protein changes in pollinated *D.* Pompdour flowers held in 0.6 mM STS solution. Visual observation of the gel (Figure 6.9A) showed that there was an increase of 30 kDa protein on day 2 until day 6. The 27 kDa polypeptide appeared to be more consistent across the gel. Lower amounts of the 15 kDa were observed, an observation similar to the rest of the protein profiles established earlier.

The observations were confirmed objectively as shown in Table 6.6. The 30 kDa protein increased on day 2 until day 6, with highest increase recorded on day 6. Based on the scores, 27 kDa protein was present in lower abundance. Where increase was measured, the value was lower than the 30kDa protein. Finally , expect for day 2, the 15 kDa protein either remained unchanged or decreased compared to day 0.

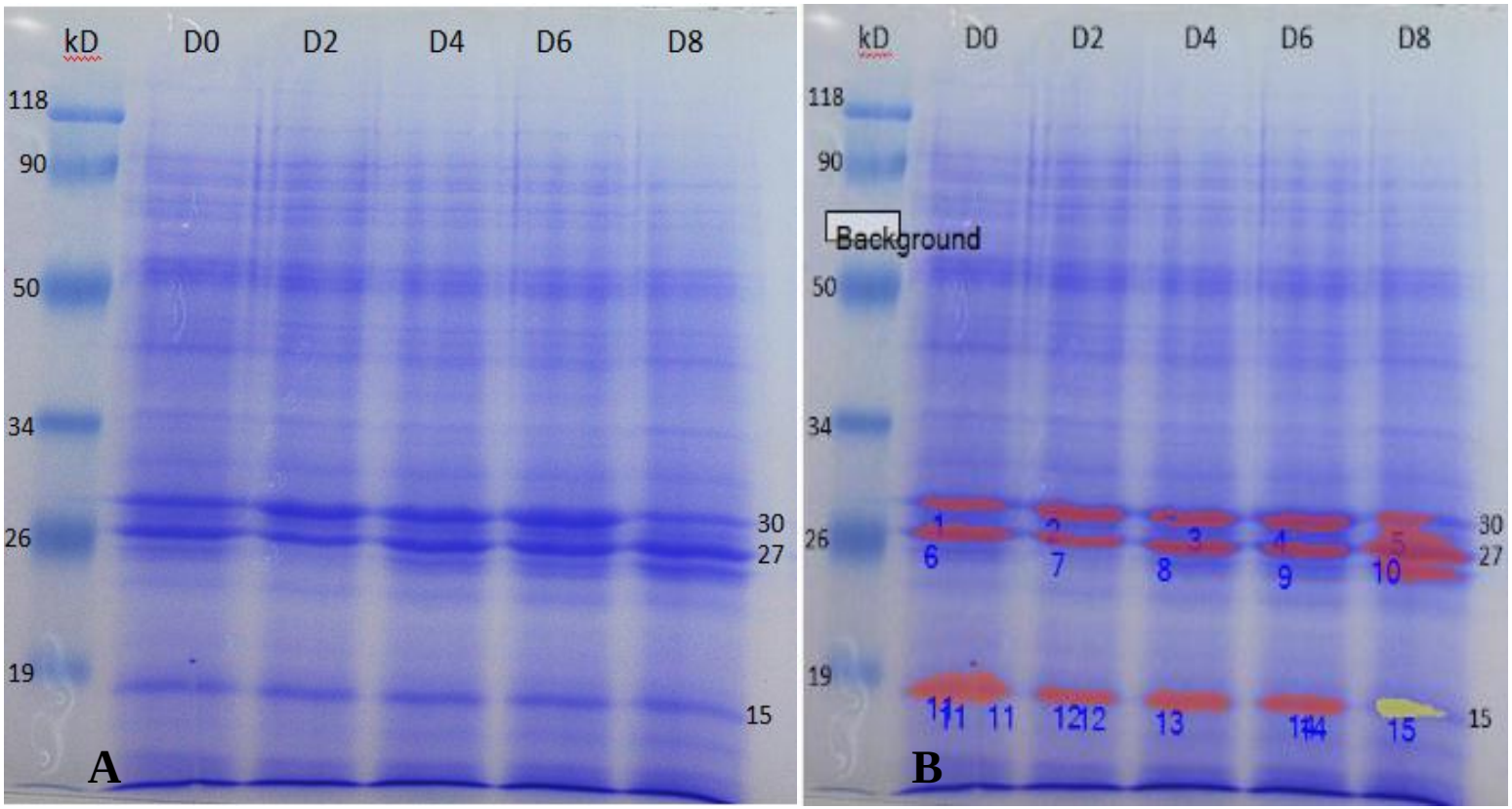

Fig 6.9 (A-B). A) Pattern of protein changes in pollinated *D.* Pompadour held in 0.6mM STS solution. Representative gel from triplicates of total soluble protein from flowers held in 0.6mM STS solution extracted every 2 days for 8 days. Proteins were separated by 10% SDS-PAGE and visualized after staining with Coomassie blue R. Each lane was loaded with 30 µg of total protein extract. Proteins with molecular masses of 15, 27 and 30 kDa were detected. Prestained Protein Molecular Weight Markers (Fermentas) are indicated on the far right. **B) Band detection image using the EZQuant 2.2 software**. The rectangle at the top right corner indicates the background against which the bands were measured.

Table 6.6 Relative abundance of polypeptides in *D.* Pompadour flowers held in 0.6 mM STS. The score is relative to the 1.0 assigned to the polypeptide expressed on day one (bands number 1, 6 and 11). The different colours indicate the different polypeptides.

Protein Size	Days	Band Number	EZQuant Value	Ratio
30kDa	0	1	237.0	1.0
	2	2	289.3	1.2
	4	3	297.6	1.3
	6	4	340.4	1.6
	8	5	264.4	1.1
27kDa	0	6	189.8	1.0
	2	7	173.4	0.9
	4	8	206.4	1.1
	6	9	199.3	1.0
	8	10	212.2	1.1
15kDa	0	11	199.6	1.0
	2	12	213.7	1.1
	4	13	206.7	1.0
	6	14	173.6	0.9
	8	15	170.2	0.9

6.3.3 N-Terminal Protein Sequence Analysis of 30kDa, 27kDa and 15kDa Polypeptides

The N-terminus of 3 polypeptides with the sizes 30 kDa, 27 kDa and 15 kDa was determined for further characterisation. The BLASTP programme provided online at NCBI home page (http://www.ncbi.nlm.nih.gov) was used to search for protein sequence homologies to proteins in the SWISSPROT database.

6.3.3.1 30kD Polypeptide

The first 11 N-terminal amino acid residues of the 30 kD polypeptide determined by protein micro-sequencing were **TKFDFFTLALQ**. Homology of the sequence and % identity to proteins in the database are shown in Table 6.7 and 6.8 respectively.

Table 6.7 Sequence homology between the 30kD polypeptide and sequences in SWISSPROT database.A comparison is made of the N-terminal sequence of *D.* Pompadour 30 kDa polypeptide to the sequences of *Engelmannia pinnatifida* (Hyunn *et al.,* 1996), *Engelmannia peristenia* (Hyunn *et al.,* 1996) *Saprolegnia monoica* (Mort Bontem, 1995), *Dictyostelium discoideum* (Zucko *et al.,* 2007) Identical polypeptides to all five sequences are in bold. The numbering is relative to the deduced translation start site.

D. Pompadour – 30kDa	**TKF**DF**F**T**LA**L**Q**	11
Engelmannia pinnatifida	**TKF**DF**F**T**LA**L**Q**	11
Engelmannia peristenia	**TKF**DF**F**T**LA**L**Q**	11
Saprolegnia monoica	**TKF**QF**F**Y**LA**L**Q**	575
Dictyostelium discoideum	**TKF**DY**F**T**LA**N**Q**	2546

Table 6.8. % Identity of the 30 kDa polypeptide to known proteins in the database

Source	% Identity
Antifungal protein of *Engelmannia pinnatifida*	100
Antifungal protein of *Engelmannia peristenia*	100
Polyketide synthase of *Dictyostelium discoideum*	83
Chitin synthase of *Saprolegniamonoica*	78

N-terminal sequencing revealed that the *D.* Pompadour 30 kDa protein had 100% identity to the N-terminus of an antifungal protein isolated from daisies approximately 80% identity to pathogen / defence related protein in other organisms. Polyketides are a diverse class of secondary metabolites that display antibiotic, antifungal, immunosuppressant and antitumor properties.

6.3.3.2 27 kDa polypeptide

The first 10 N-terminal amino acid residues of the 27 kDa polypeptide determined by protein micro-sequencing were **SGVGHVPSTT**. Homology of the sequence and % identity to proteins in the database are shown in Table 6.9 and 6.10 respectively.

Table 6.9 Sequence homology between the 27 kDa polypeptide and sequences in SWISSPROT database.A comparison is made of the N-terminal sequence of *D.* Pompadour 27 kDa polypeptide to the sequences of *Methanococ cusmaripaludis C5*, *Methanococ cusaeolicus Nankai-3* (Copeland *et al.,* 2007),*Methanopyrus kandleri AV19* (Slesarev *et al.,* 2005)and *Halobacterium sp* (Ng *et al.,* 2000). Identical polypeptides to all five sequences are in bold. The numbering is relative to the deduced translation start site.***** indicates gap introduced to aid the alignment

D. Pompadour – 27kDa	**S*GVG**HV**P***ST**T**	10
Methanococcusmaripaludis	**SFGVG**HA**P**LST**T**	176
Methanococcusaeolicus	**SFGVG**HA**P**FST**T**	175
Methanopyruskandleri AV19	**SFGVG**YA**P**LTT**T**	170
Halobacterium sp.	**SFGVG**HA**P**LTE**T**	129

Table 6.10 % Identity of the 27 kDa polypeptide to known proteins in the database

Source	% Identity
SAM synthase of *Methanococcus maripaludis C5*	80
SAM synthase of *Methanococcu saeolicus Nankai-3*	80
SAM synthase of *Methano pyruskandleri* AV19	70
SAM synthase of *Halobacterium* sp.	60

N-terminal sequencing revealed that the *D.* Pompadour 27 kDa protein had more than 60% identity to the N-terminus of SAM synthases isolated from a number of microbes. SAM synthase is the enzyme that catalyses the conversion of methionine to S-adenosylmethionine in the Yang cycle.

6.3.3.3 15 kDa polypeptide

The first 13 N-terminal amino acid residues of the 15 kDa polypeptide determined by protein micro-sequencing were **MVIVLVVWLALSA**. Homology of the sequence and % identity to proteins in the database are shown in Table 6.11 and 6.12 respectively.

Table 6.11 Sequence homology between the 15kDa polypeptide and sequences in SWISSPROT database. A comparison is made of the N-terminal sequence of *D.* Pompadour 15 kDa polypeptide to the sequences isolated from *Zea Mays* (Marks *et al.*, 1985), *Sorghum propinquum* (Laidlaw *et al.*, 2010), S*orghum bicolor* (Laidlaw, 2010) and *Coixlacryma*-jobi (Leite, 1991). Identical polypeptides to all three sequences are in bold. The numbering is relative to the deduced translation start site.

*D.*Pompadour – 15kDa	**MVIVL**V**V**W**LALSA**	13
Zea mays	**MVIVL**V**V**W**LALSA**	15
Sorghum propinquum	**MVIVL**A**V**C**LALSA**	15
Sorghum bicolor	**MVIVL**A**V**C**LALSA**	15
Coixlacryma-jobi	**MVIVL**A**V**C**LALSA**	15

Table 6.12. % Identity of the 15 kDa polypeptide to known proteins in the database

Source	**% Identity**
beta zein precursor of *Zea* mays	100
beta kafirin precursor of *Sorghum propinquum*	92
beta kafirin of *Sorghum* bicolor	92
Alpha coixin of *Coixlacryma*-jobi	92

N-terminal sequencing revealed that the *D.* Pompadour 15 kDa protein had more than 90% identity to the N-terminus of seed storage proteins isolated from a number grains and grass. Zein, kafirin and coixin are all proteins in the prolamin family, characterised by a high glutamine and proline content and are generally soluble only in strong alcohol solutions.

6.4 DISCUSSION

The results in this chapter show that total protein in flowers undergoing natural aging (unpollinated) was significantly different compared to those undergoing pollination-induced senescence. Unpollinated flowers exhibited no change in total protein content within eight days of observation. In contrast rapid decrease of protein content was evident in pollinated flowers. This confirms that *D.* Pompadour flowers follow the same pattern of protein loss following pollination as observed in other orchids such as *Phalaenopsis amabilis, D. Nobile, Cattleya labiata* (Schumake, 1931) and *D.* Khao Sanan (Lerslerwong *et al.,* 2009). Drastic decline in protein content prior to wilting have also been reported in other flowers such as *Ipomoea, Hemerocallis* and *Petunia* (Matile and Winkenbach, 1971; Lay-yee *et al.,* 1992; Sultan and Farooq, 1996; Gulzar, 2003).

A number of events may be causing this decrease. The loss in wall proteins could be one. As observed in chapter 3, pollinated flowers exhibited rapid flower closure and a week after pollination the perianth appeared shrivelled with signs of necrosis and browning. Furthermore thinning of petals also occurred. All these morphological changes are related to destruction of cell wall integrity and structure. Although cell wall and membrane are made primarily of polysaccharides, wall proteins are cross-linked to the wall and serve an integral part in cell structure (Cassab, 1998).

Loss in proteins may also be caused by hydrolysis. Previous chapters have discussed that pollination- induced senescence is a phenomenon that involves hydrolysis and export of materials, which also includes proteins. A rapid increase of protease activity was observed in pollinated *Arachnis* orchid blossoms (Hew *et al.*, 1989). In pollinated *Cymbidium pendulum* and *Cymbidium aloifolium*, protease activity increased in the lip

and the perianth but remained relatively unchanged in the ovary. This observation is indicative of the rapid breakdown of proteins in senescing floral segments following pollination (Attri *et al.,* 2008).

In other flowers such as carnations (Woodson and Handa 1987), *Geraldton* waxflowers (Olley *et al.,* 1996), *petun*ia (Xu and Hanson, 2000), and rose (Mayak, 1976) protein degradation is a common occurrence during senescence. During shortages of respirable substrates, amino acids are converted into glucose which is used in existing metabolic processes. Himelblau and Amasino (2001) stipulated that nitrogen is the primary nutrient released during protein breakdown and suggested that it is the most recycled nutrient during senescence accounting for approximately 90% of total nutrients recycled. One of the most important characteristics of programmed cell death such as pollination induced senescence is the remobilization of nitrogenous substances in the form of amino acids to developing tissues (Soudry *et al.,* 2005).

For example, after pollination of *Cattleya labiata* (Hsiang, 1951) and *Arachnis* Maggie Oei (Hew *et al.,* 1989), total nitrogen content of the perianth decreased while that of the gynostemia and ovaries increased. In senescing floral segments of *Arachnis* Maggie Oei, amino acid content was found to decrease while continuously increasing in both the ovary and gynostemium (Hew *et al.,* 1989).

Total protein is made up by insoluble and soluble proteins. Both these proteins show different patterns in pollinated *D.* Pompadour flowers. This may indicate that pollination induces change on insoluble rather than soluble proteins. While soluble proteins remained unchanged, insoluble proteins exhibited a rapid decrease. This observation is similar to that reported in *Dendrobium* Khao Sanan and *Dendrobium*

Sonia Bom #17 orchids where decrease in insoluble proteins preceded visible symptoms of senescence. Exposure to ethylene did not result in any change to the level of soluble proteins (Lerslerwong *et al.,* 2009). The authors suggested that the loss in some soluble proteins may be compensated by new synthesis of proteins and enzymes and possibly by the transfer of insoluble proteins into the soluble fraction. Matile and Winkenbach (1971) explained that while peptidase activity increases, increase in synthesis of new enzymes also occurs at the same time, most of which are hydrolytic in nature. Proteomic studies on pollinated petunia revealed the increase of soluble proteins involved in catalysing carbohydrate breakdown, proteolytic processes, cell and nucleic acid degradation (Bai *et al.,* 2010).

Insoluble proteins are predominantly localised at the membrane, therefore the net decrease of these proteins may be largely due to loss in membrane proteins. This supports the correlation between loss of protein with destruction of flower integrity and structure as discussed earlier. Insoluble proteins may also be used as respirable substrates as reported by Hooshdaran *et al.*, (2004). Furthermore, some existing protease catalyse the degradation of insoluble proteins (Wagstaff *et al.,* 2002; Schaller, 2004).

Both the 0.05 mM AOA and 0.6 mM STS treatment solutions resulted in protein levels that were similar to that of unpollinated flowers. Total protein content was higher than pollinated flowers held in water and remained unchanged throughout the experiment. This observation suggests that protein degradation may be regulated by ethylene during pollination-induced senescence, at least in *D.* Pompadour orchids. In carnations, the rapid induction of ethylene following pollination was concomitant with the rise in cysteine protease expression. This indicated the increase in protein breakdown during

the climacteric production of ethylene. Furthermore, treatment with an ethylene inhibitor, NBD prevented the increase in cysteine protease transcription (Jones *et al.*, 1995). Similarly, pre- treatment with STS significantly reduced the levels of protease activity in *Alstromeria* (Wagstaff *et al.*, 2005).

In this chapter, the use of 4% glucose and 2% sucrose managed to delay protein loss. These sugars however were not as effective as the ethylene inhibitors. Unlike the flowers treated with ethylene inhibitors where protein levels remained unchanged, *D.* Pompadour treated with sugar exhibited a significant decline in total protein 6 days after pollination. The reasons for this have been discussed earlier in Chapter 3. Exogenous sugars have been reported to delay proteolysis and promote protein synthesis (Paulin, 1986). In senescing broccoli florets, retention of protein was evident in flowers treated with sucrose. At the same time, increase in protease activity was delayed. Cystein protease was found to accumulate at a slower rate following treatment of *Sandersonia* tepals with sucrose, coupled with a higher retention of soluble protein. These observations suggest that there may be some transcriptional regulation of proteases by sucrose (Coupe *et al.*, 2003). Another possibility could be related to metabolite breakdown. As was observed in the previous chapter, sugar content in treated flowers remained stable throughout the experiment. This may lead to retention of proteins as breakdown of secondary metabolites was not necessary.

In order to investigate the pattern of protein changes during pollination induced senescence, 1D SDS PAGE analysis was carried out. The data presented in this chapter revealed changes in protein expression following pollination. Three polypeptides with sizes 30 kDa, 27 kDa and 15 kDa were identified and characterised. N terminal sequencing and subsequent homology analysis showed that the 30kDa protein shared high homology with pathogenesis-related (PR) proteins. These included a putative

antifungal protein and enzymes that catalyse the production of chitinase and polyketide. SDS PAGE of all treatments and control indicated the presence of PR proteins. However, the expression of these proteins was found to be the most abundant in pollinated flowers 6 and 8 days after pollination.

At this point the perianth has reached a necrotic state. The substantial increase in the expression of these PR proteins implicates the important role of these proteins following pollination. Pollination leads to the subsequent penetration of the aseptic pollen tubes which in turn wounds the pistil (van Eldik *et al.,* 1995). Furthermore, metabolic resources are rapidly broken down to be utilized by the developing ovary. Taken together, the enriched nutrient and wound offer a suitable environment for pathogens. It is therefore imperative for pollinated flowers to produce compounds that protect the developing ovary and the seed (Day and Tuite, 1998; Buchanan *et al.,* 2000).

In pollinated *Petunia* blossoms, 2D proteomic analysis showed that the largest number of upregulated proteins were defence –related genes, with 64-66% of all the upregulated proteins belonging to that category (Bai *et al.,* 2010). In apples, a large number of PR genes were found to be induced by pollination. One of that is the chitinase which increased substantially following pollination. The expression of these genes occurred 48 hours after pollination, long before fertilization occurs (Williams, 1965). Furthermore, pistil-specific PR1 proteins and floral defensins have both been reported to be expressed in potato and tobacco flowers (Lay *et al.,* 2003).

It is important to note that in this chapter, increase in the PR proteins was also evident in all flowers, although not as high as those expressed in pollinated flowers. This may be a response to the environment stress all these flowers were exposed to as they were cut from the mother plant. However, in accordance with the ethylene-induced nature of

PR proteins, the most abundant expression of these proteins coincided with the production of climacteric ethylene in pollinated flowers. This observation lends support to previous works that reported the need for ethylene to induce the expression of PR proteins. In fact, enhanced ethylene production is an early, active response of plants to perception of pathogen attack and is associated with the induction of defence reactions. A number of studies have revealed that senescence and PR proteins are linked via an ethylene dependant signaling pathway (Greenberg and Ausubel, 1993; Chen and Bleecker, 1995; Larson and Chang, 2001; Passarinho and Vries, 2002). This however does not exclude the regulation of PR proteins by other hormones such as methyl jasmonate and gibberellins (Wu and Bradford, 2003).

In view of the relation that may exist between ethylene and the induction of PR proteins, it is of no surprise that flowers held in both 0.05 mM AOA and 0.6 mM STS resulted in lower amounts of PR proteins compared to non-treated flowers. In their attempt to establish the involvement of ethylene in the induction of PR proteins, many researchers have turned to ethylene inhibitors. A study carried out by Ohtsubo *et al.*, (1999) confirmed this by demonstrating the decrease of PR protein expression in TMV infected leaves treated with ethylene biosynthesis and action inhibitors. In bacteria infected pepper leaves, treatment with AVG (an ethylene biosynthesis inhibitor) strongly suppressed the induction of PR-1mRNA. This led to the speculation that ethylene may act as a strong signal elicitor on the activation of PR-1 genes during plant-pathogen interaction (Kim and Hwang, 2003).

On the other hand, it has long been established that plants have long evolved a mechanism to modulate sugar levels in response to pathogenenesis (Watson and Watson, 1951; Wolfe *et al.*, 2000). Altered levels of sucrose in rice plants were found to act as signals to activate defence genes (Jorge *et al.*, 2007). It is therefore reasonable to assume that the increase in PR proteins of *D.* Pompadour flowers held in 4% glucose and 2% sucrose is a result of some form of endogenous sugar signalling.

The 27 kDa polypeptide was identified as SAM synthase and shared more than 60% identity to the N-terminus of SAM synthases isolated from a number of microbes. Protein profiles of all flowers in this chapter showed the presence of SAM synthase, the enzyme that catalyses the conversion of methionine to SAM in the ethylene biosynthesis cycle. The constitutive presence of this protein could be explained by a couple of reasons. First, ethylene is still present albeit at very low levels unpollinated flowers and treated flowers. Second, besides being a substrate in the ethylene pathway, SAM is also the major donor in transmethylation reactions of DNA, RNA, lipids as well as being intermediates for polyamine and biotin synthesis (Roeder, 2009). Nevertheless the data in this study demonstrated that a significant increase in the expression of SAM synthase protein was evident in pollinated flowers. In unpollinated flowers and treated flowers insignificant change in the protein was observed while in pollinated flowers, increase was almost two folds. This significant change suggests SAM synthase may be play an important role in the ethylene production pathway during pollination-induced senescence.

To date, very little data is available with regards to the expression of SAM synthase in plants following senescence. Existing literature reports conflicting data in terms of spatial and temporal expression of SAM synthase in plants. For example, while SAM synthase was expressed in the styles and stigma of *Nicotiana suaveolens* (Roeder, 2009), none was detectable in the inflorencses of *Arabidopsis thaliana* and *Pinu scontorka* (Lindroth *et al.,* 2001). Furthermore, Woodson *et al.,* (1992) postulated that increase in ethylene is not dependant on the availability of SAM. This was evident in the decrease of mRNA levels of SAM -2 in senescing carnations. In contrast a number of evidence suggests a strong correlation between ethylene and SAM. For instance, transgenic tomatoes with degraded SAM exhibited 60-80% decrease in ethylene production. In mature and immature kiwi fruits, exogenous ethylene was found to induce the expression of SAM synthase (Whittaker *et al.,* 1997). It is therefore reasonable to suggest that the increased expression of SAM synthase maintains the SAM levels under conditions of high ethylene concentration.

The findings presented in this chapter indicate that there is a need for the cell to stabilize the levels of SAM following ethylene production during pollination induced senescence despite ACC being the rate limiting step. Furthermore, the absence of increased SAM synthase protein in flowers treated with 0.05 mM AOA and 0.6 mM STS further supports the ethylene dependant translation of the SAM synthase polypeptide.

The third polypeptide identified in this chapter showed homology to zein, kafirin and coixin proteins found in maize, cereal and grass respectively. All of which are part of the prolamin family, a group of hydrophobic, alcohol-soluble reserve glycoproteins constituting 40-60% of total seed protein (Adelli and Altosar, 1984). In flowers, although prolamins are present, the more predominant reserves consist of lipid. This is also the case with *D.* Pompadour where 1D SDS PAGE protein profile of all flowers show that these proteins are present in significantly lower abundance compared to the PR and SAM synthase polypeptides. Since orchid seeds are the smallest with most weighing between 0.3-14µg, the energy reserve is insignificant compared to larger seeds. Furthermore, most of the time during orchid seed development; nutrients are derived from mychorizal fungi, from which they form a symbiotic relationship with (Manning and Van Staden 1987; Richardson *et al.*, 1992; for review see Arditti *et al.*, 1994). In spite of the low amounts of reserve proteins, initial increase in the reserve protein expression was still observed in pollinated flowers 2 days after pollination. It has been presumed that accumulation of reserve materials reflects the progression of ovary development and the increase in metabolic rate that ensues (Bandal and Malik, 1979).

Data obtained from the protein profile of pollinated flowers indicate that the increase in storage protein coincides with the onset of ovary growth in *D.* Pompadour as reported by Ketsa and Rugkong (1999) where swelling of the ovary began 2 days after pollination. In unpollinated flowers where no change in storage protein was observed, it could be assumed that there is no necessity for accumulation of materials as ovary development is absent.

Flowers held in sugars and ethylene inhibitors did not show any increase in the expression of storage protein throughout the experiment. This demonstrates that there may be a correlation between ethylene and seed storage protein expression. A study on zein protein regulation showed that addition of methionine resulted in the accumulation of the storage protein in maize (Amira *et al.*, 2005). This study was not conclusive enough to prove the role of ethylene in zein protein regulation as methionine is also involved in other cycles. However, Gallie and Young (2004) suggested that ethylene was in fact synthesised in the ovary and endosperms. In a number of studies however methyl jasmonic has been implicated in the regulation of seed storage proteins instead (Staswick, 1990; Anderson, 1991; Koda, 1992). Nevertheless, judging from the low levels of seed storage proteins in all the SDS PAGE profiles, we could speculate that the proteins may not play a major role during pollination- induced senescence.

In conclusion this chapter shows that pollination affects protein status in *D.* Pompadour by inducing decrease in total and insoluble protein content. PR proteins and SAM synthase are present in abundance and are increased following pollination. Seed storage protein is also increased by pollination but is not as abundant as PR proteins and SAM synthase. The exact roles of the proteins have yet to be elucidated by this study. Specific 2D proteomic studies could reveal greater number of proteins that are of importance during pollination-induced senescence.