Daylily (*Hemerocallis*) as a model system for the study of ethylene-insensitive flower senescence: tissue culture and aspects of the development of proteolytic enzyme activities, with special emphasis on leucine aminopeptidase.

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To my father who to me was a beacon of great achievement

And

To my mother for laying my course this far,

I gratefully dedicate this.

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ABBREVIATIONS

ABA	abscisic acid
ACC	1 aminocycloprpopane-1-carboxylic acid
$AgNO_3$	silver nitrate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BA	benzylaminopurine
BES	bestatin
β-NA	β-naphthylamide
Ca^{++}	calcium ions
cDNA	complementary DNA
cv.	cultivar
cvs.	cultivars
2,4-D	2,4-dichlorophenoxyacetic acid
DEAE-	diethylaminoethyl-
DIG	digoxygenin
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
GA_3	gibberellic acid
HCl	hydrochloric acid
IAA	indole-3-acetic acid
IAM	iodoacetamide
IBA	indolebutyric acid
IEF	isoelectric focussing
2iP	isopentylaminopurine
KCl	potassium chloride
kDa	kilodaltons
kPa	kilopascal
LAP	leucine aminopeptidase
lap	DNA or RNA encoding leucine aminopeptidase
LB	Luria-Bertani medium
Mg^{++}	magnesium ions
$MgSO_4.7H_2O$	magnesium sulphate (hepta hydrate)
MS	Murashige and Skoog medium
Mn^{++}	manganese ions
$MnSO_4.4H_2O$	manganese sulphate (tetra hydrate)
mRNA	messenger RNA
NAA	α-naphthaleneacetic acid
NaCl	sodium chloride
Na_2HPO_4	dibasic hydrogen phosphate
NaOH	sodium hydroxide
$Na_2S_2O_3.5H_2O$	sodium thiosulphate
NEM	N-ethyl-maleimide
$(NH_4)_2SO_4$	ammonium sulphate
PAGE	polyacrylamide gel electrophoresis
pCMB	ρ-chloromercuribenzoate

PEG	polyethylene glycol
PGSPs	preglobular stage proembryos
PHMB	p-hydroxymercuribenzoate
pHMB	p-hydroxymercuribenzoate
PMSF	phenylmethylsulfonylfluoride
p-NA	ρ-nitroanilide
RNA	ribonucleic acid
RNAase	ribonuclease
rRNA	ribosomal RNA
STS	silver thiosulfate
SDS	sodium dodecyl sulfate
SH-reagent	sulfhydryl reagents
TEMED	N,N,N',N'-tetramethylethylenediamine
UV	ultra violate light
Zn^{++}	Zinc ions
$ZnSO_4.4H_2O$	Zinc sulphate (hepta hydrate)

ABSTRACT

A micropropagation technique has been developed using petal and ovary explants from floral buds of several daylily cultivars. The growth regulators NAA and BA or 2iP in ½ strength MS was used for the initiation of shoot primordia while rooting was carried out in growth regulator-free ½ strength MS. Many plantlets of cv. Stella D'oro were grown successfully in the glasshouse to supply flowers for the experiments on senescence in this study.

The role of proteolytic enzymes with special emphasis on leucine aminopeptidase (LAP), in senescence of ethylene-insensitive flowers was studied using daylily flowers. During bud development the LAP activity began to increase and reached a maximum 6 hr after the flower was fully open. Increase in endopeptidase activity began only after the flower was fully open and reached a maximum value 12 hr later. As the flower senesced, both enzymes decreased. In contrast, carboxypeptidase level remained constant throughout flower development. Therefore, LAP appears to be responsible, at least for the initiation of proteolysis during senescence of petals, while endopeptidase may be responsible for the massive proteolysis that occurred during later stages of senescence. Carboxypeptidase on the other hand does not seem to play a special role during senescence. There was no evidence for the presence of endogenous inhibitors or promoters against the 3 proteolytic enzymes studied here nor the occurrence of low-pH extractable forms of the 3 enzymes in daylily petals.

When experiments were carried out to determine the effect of numerous chemicals on vase life of daylily flowers, cycloheximide was the only chemical that delayed senescence of daylily flowers. Cycloheximide treatment also retained the high levels of LAP and *lap* mRNA associated with full bloom flowers. In contrast to flowers, several chemicals including ethrel, cycloheximide, BA, sucrose, fructose were effective in retaining the green colour of daylily leaf discs for a longer time than those kept in water while ABA accelerated yellowing of leaf discs. GA_3 kept the green colour of the leaf discs a little longer than water controls. Silver thiosulfate, ascorbic acid, casein hydrolysate, glyphosate, kinetin and IAA caused yellowing of leaf discs around the same time as water controls. 8-hydroxyquinoline caused browning of leaf discs, so did the glucose in 8-hydroxyquinoline. Green leaf discs treated with cycloheximide and ethrel had lower levels of LAP than the yellow ones in water. Yellow leaf discs in ABA also had lower levels of the enzyme than the green ones in water. Yellow leaf discs in silver thiosulfate had similar levels of LAP to those kept in water. In contrast to flower senescence, yellowing of daylily leaf discs was not always accompanied by a reduction in LAP. Therefore, LAP appears to have different roles during senescence of leaves and flowers.

IEF gel analysis revealed that daylily extracts had 2 closely run LAP isozyme bands of pI 5. LAP in daylily is a thermolabile, metallo enzyme, whose activity was enhanced by Mn^{++}. EDTA, PMSF, NEM and Zn^{++} inhibited enzyme activity while Mg^{++} and leupeptin had no effect. It also did not need SH-protecting agents for activity. The optimum pH for the enzyme activity was 8.

Dot blot hybridization of mRNA from daylily petals using *lap* cDNA probe from *Arabidopsis thaliana* showed that LAP in daylily petals is under developmental regulation. Petals from buds and full bloom flowers of daylily had equal amounts of *lap* mRNA while those from senesced flowers had very low amounts of *lap* mRNA.

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CHAPTER 1

INTRODUCTION

1.1 SENESCENCE OF PLANTS AND PLANT PARTS

Senescence may be defined as the endogenously controlled deteriorative processes that lead to death of cells, tissues or organs (Noodén, 1988a). The main characteristic of cell death seems to be the loss of its ability to maintain homeostasis. In thermodynamic terms, cells die when they cannot maintain their entropy below that of their environment (Noodén, 1988a). Maintaining the cells entropy requires the integrity of membranes as well as a constant input of energy.

Senescence in plants shows a wide range of patterns, involving either the entire plant or separate plant organs. Senescence could result in the total death of the entire plant at the end of the reproductive phase, death of all above-ground organs, death of the entire array of leaves at the end of the growing season and progressive senescence of leaves along the stem (Thimann, 1980). Even in a single plant senescence takes place in different ways. For instance, in corn (*Zea mays* L.), canopy senescence occurs in two phases, foliar symptoms are first observed in the bottom leaf and in sequential order up the plant. Subsequently, senescence occurs on the top leaf and moves downward (Feller et al., 1977).

Senescence has positive values in terms of ecological adaptation, natural selection and efficiency of internal physiology (Leopold, 1961). Annuals have adapted to grow and reproduce during the best period for their growth and die thereafter, avoiding intense competition and harsh environmental conditions. Senescence of the whole plant is a catalyst for evolutionary adaptability (Leopold, 1961). A

population of perennial plants that have a long life span will continue to reproduce its original genome for a long period, whereas in a population of annuals, overall senescence will impose a rapid turnover of individuals that are more adapted to new environmental niches.

Senescence of mature, functioning, intact organs is a unique feature restricted to plants only. This may have evolved to remove the parts that are expensive to maintain and are no longer required or functional. For example, flowers senesce once they are pollinated, while leaves senesce when they are shaded or when the winter is approaching. Another advantage of organ senescence is seen in fruits. During senescence, fruits become more attractive to animals, assisting dissemination of seeds. In addition spread of seeds may be facilitated by the softening of the pericarp and dehiscence of seams of the fruit.

Even though senescence has been defined as an irreversible process (Sacher, 1973), there is evidence that symptoms of senescence can be reversed in plant parts before senescence has proceeded beyond a certain stage at which it becomes irreversible (Wittenbach, 1978; Wulster et al., 1982; Woodson et al., 1985).

1.1.1 Senescence Symptoms

Many ultrastructural, biophysical and biochemical changes are associated with senescence leading to cell death. Although not yet clearly understood, some of these changes could be the cause of senescence while others could be the result. The changes that are taking place during the process of senescence plants and plant organs are described in the following sections.

1.1.1.1 Changes in Fresh and Dry Weights

In most plant organs, weight is maintained more or less constant until the last stages of senescence. During senescence fresh weight may decrease (Lay-Yee et al., 1992; Bieleski, 1993; Eason and Webster, 1995; Collier, 1997) or remain unchanged (Stead and Moore, 1977) while dry weight may decline (Stead and Moore, 1977; Lay-Yee et al., 1992; Collier, 1997). Loss of fresh weight during senescence is mainly due to loss of water from tissues while loss in dry weight could be a result of loss of moisture and also loss of macromolecular components.

1.1.1.2 Changes in Pigmentation

In most organs, senescence is accompanied with a change in coloration. Yellowing of leaves or reduction in greenness is one of the main features of leaves undergoing senescence (McHale and Dove, 1968; Thimann, 1980; Gepstein, 1988). Change in coloration also occurs during fruit ripening and flower senescence (Eason and Webster, 1995) with some exceptions (Stead and Moore, 1977).

1.1.1.3 Changes in Membranes and Fine Structures

Cellular membranes are selective, dynamic barriers that play an essential role in regulating biochemical and physiological events (Thompson, 1988). During senescence there is a progressive loss of membrane integrity, which is shown by ultrastructural studies showing progressive deterioration of organelles and membranes, and also by permeability studies indicating increased leakage of solutes. There is also increasing evidence for subtle changes in the molecular organisation of deteriorating membranes that result in an orderly and progressive

loss of functional and structural integrity. In ageing rose petals and protoplasts isolated from rose petals there was an increase in microviscosity of plasma membranes (Borochov et al., 1976; 1978), together with increases in sterol to phospholipid ratios. This change in membrane fluidity was a result of a decrease in phospholipid content, brought about by both reduced synthesis and enhanced degradation (Borochov et al., 1982).

In green tissues, some of the earliest changes in senescence are visible in chloroplasts. The early changes in chloroplasts include swelling of the thylakoids and appearance of lipid droplets and plastoglobuli (Noodén, 1988a). Loss of ribosomes from cytoplasm and chloroplast is also an early event of senescence. As senescence advances there is loss in integrity of the plasmalemma, and mitochondrial and vascular membranes. During this time autophagic processes come into action causing organelles to be enveloped in vacuole-like structures (Matile and Winkenbach, 1971).

1.1.1.4 Changes in Macromolecular Components

1.1.1.4.1 Hydrolysis and translocation of cell components

With the loss of membrane structure in cells undergoing senescence, hydrolytic enzymes that have been compartmentalised in the cell are released (Matile and Winkenbach, 1971) which, causes massive breakdown of cellular components (Kenis et al., 1985). There is evidence to support that these products of hydrolysis are mobilised to other parts of the plant (Suttle and Kende, 1980). Redistribution of assimilates and products of hydrolysis to developing fruits had been considered to play a causal role in monocarpic senescence (Nooden, 1988b). Translocation of soluble sugars and amino acids from senescing petals to other developing parts is

seen in some flowers (Nichols and Ho, 1975; Bielesky, 1995), although it is relatively non-significant in some other flowers (Collier, 1997).

1.1.1.4.2 Proteins

In tissues undergoing senescence there is a general decline in protein levels. One of the main reasons for this is the large-scale proteolysis of cellular proteins (Makrides and Goldwaite, 1981; Lay-Yee et al., 1992; Eason and Webster, 1995; Collier, 1997). However, reduced synthesis of proteins may also contribute to this reduction in protein levels (Leshem et al., 1986). Although there is a general trend towards reduction in total proteins in tissues undergoing senescence, there is evidence for the synthesis of certain proteins also (Eason and de Vré, 1995). In detached, yellowing barley leaves, total ^{14}C-alanine incorporation was enhanced by about 20% within 6 hr of leaf detachment and by about 30% 24 hr later (Atkin and Srivastava, 1970). Furthermore, external application of protein synthesis inhibitors such as cycloheximide (Martin and Thimann, 1972; Lay-Yee et al., 1992; Bieleski, 1993; Jones et al., 1994; van Doorn et al., 1995; Burge et al., 1996) and RNA synthesis inhibitors such as actinomycin D (McHale and Dove, 1968) had delayed senescence of plant organs. Some of these newly synthesised proteins have protease activity (Martin and Thimann, 1972; Woodson, 1987).

1.1.1.4.3 Nucleic acids

In tissues that are in the process of senescence a considerable decrease in both DNA and RNA has been observed where RNA decreases at a faster rate than DNA. In bean leaves both cytoplasmic and organelle rRNA declined during maturity and senescence (Makrides and Goldwaite, 1981). Reduction in RNA levels may result from increased activity of RNAase that breaks down RNAs or from reduced activity

of RNA polymerase that synthesise them. There is substantial evidence for an increase in RNAase activity in plants and organs undergoing senescence while very little is known about RNA polymerase enzyme in those plants and organs (Lesham et al., 1986). In tomato leaves, application of actinomycin D inhibited the natural increase in RNAase activity, seen in ageing leaves, indicating that RNAase is newly synthesised in that tissue (McHale and Dove, 1968). There was an increase in RNAase activity in excised *Avena* leaves and in *Ipomoea* corolla during senescence (Udvardy et al., 1969; Matile and Winkenbach, 1971).

Although there is reduction in total RNAs, there are many RNAs that increase during senescence (Woodson, 1987; Woodson and Lawton, 1988). These are the mRNAs that encode for the proteins synthesised during senescence (Biggs et al., 1986; Wang et al., 1993).

There are few studies on the amount of DNA in tissues undergoing senescence. These studies show that DNA levels could vary among different species and organs. For example, in bean leaves the amount of total DNA remained unchanged even after abscission and withering of lamina (Makrides and Goldwaite, 1981). In contrast, nuclear DNA in soybean cotyledons was reduced by 23% during senescence due to selective degradation of some repetitive DNA (Chang et al., 1985). At the onset of *Ipomoea* corolla wilting there was a dramatic increase in DNAase activity, which would lead to a reduction in the DNA level (Matile and Winkenbach, 1971).

1.1.1.4.4 Carbohydrates

Senescence in plant organs is accompanied by hydrolysis of starch, sugars and cell wall polysaccharides (Matile and Winkenbach, 1971; Tetley and Thimann, 1974; Hawker et al., 1976; Warman and Solomos, 1988). This is evident by increases in

carbohydrate degrading enzymes in organs undergoing senescence (Tucker and Grierson, 1982; Slater et al., 1985; Panavas et al., 1998a).

Usually flower senescence is characterised by a decline in carbohydrate content (Ho and Nichols, 1977; Stead and Moore, 1977; Collier, 1997; Bieleski, 1993). For this reason, soluble carbohydrates, mainly sucrose is often added to preservative solutions to extend vase life of flowers (Halevy and Mayak, 1981). In flowers of *Leptospermum scoparium* Forst., vase life was greatly improved when sucrose (30-60 g/L) was provided with of 8-hydroxyquinoline citrate (Burge et al., 1996). The vase life of flowers of *Gloriosa* and *Gladiolus* spp., had also been enhanced by application of sucrose as a pulse or continuous treatment (Bravdo et al., 1974; Jones and Truett, 1992; Serek et al., 1994; Eason et al., 1997). Exogenous application of sugars is thought to maintain osmotic potential, respiration rate and membrane integrity of tissues (Halevy and Mayak, 1979).

1.1.1.5 Changes in Respiration

In some plant organs, senescence is accompanied by a sharp increase in respiration. For instance, in detached fruits such as apples, pears and bananas there is a brief but significant rise in respiration at some stage during ripening before irreverisible breakdown of the tissues (Lesham et al., 1986). This rise in respiration is termed 'climacteric'. Alternatively, there are 'non- climacteric' fruits such as citrus fruits, pepper and grape that do not show such a marked increase in respiration during senescence. Similar to fruits, senescing flowers are also known to show two patterns of respiration (see section 1.1.3). Most of the leaves too have been shown to manifest climacteric behaviour (Smillie, 1962; Tetley and Thimann, 1974; Malik and Thimann, 1980; Warman and Solomos, 1988).

1.1.1.6 Changes in Photosynthesis

In leaves, a decline in photosynthesis is a common feature of senescence (Smillie, 1962; Thimann, 1980). This is associated with the disappearance of chlorophyll (Goldwaite and Laetsch, 1968; McHale and Dove, 1968; Tetley and Thimann, 1974) and disintegration of chloroplasts (Lesham et al., 1986). Also, there is considerable evidence for the loss of ribulose biphosphate carboxylase, a major enzyme involved in photosynthesis (Wittenbach, 1978; 1979). However, a decline in photosynthesis is not regarded as the senescence-triggering mechanism, because albino leaves and flower petals that do not contain chlorophyll senesce under similar conditions to those that cause senescence of leaves (Lesham et al., 1986).

1.1.1.7 Changes in Plant Hormones

Members of each of the five groups of natural plant hormones and their synthetic counterparts have been implicated in the regulation of senescence. However, ethylene seems to be the most extensively studied plant hormone in relation to senescence. Organs undergoing senescence could behave in two different ways as far as ethylene production is concerned; those that show an ethylene production peak and those that do not produce such a peak during senescence. With a few exceptions (Lukaszewski and Reid, 1989; Bieleski and Reid, 1992; Serek et al., 1994) most of the plant organs undergoing senescence are accompanied by a respiratory peak show an increase in ethylene production as well. These include climacteric fruits, ethylene-sensitive flowers (see below), leaves (Warman and Solomos, 1988) and whole plants. Exogenous application of ethylene, ethylene-like substances (propylene), precursors of ethylene (1 aminocyclopropane-1-carboxylic acid i. e. ACC) and compounds that release ethylene (ethrel, 2-chloroethylphosphonic acid) is known to enhance the symptoms of senescence in such tissues (Nichols, 1971; Mor et al., 1980; Reid et al., 1980; Suttle and Kende,

1980; Wulster et al., 1982; Whitehead et al., 1984a and b; Warman and Solomos, 1988). On the other hand application of inhibitors of ethylene synthesis (aminooxyacetic acid or aminoethyoxyvinylglycine) and ethylene action (silver thiosulfate i.e. STS or 2,5-norbornadine) can delay such symptoms (Whitehead et al., 1984a). Plant organs that do not show an ethylene peak during senescence do not respond to the above mentioned chemicals (Eason and De Vré, 1995).

Although ethylene has been suggested to play a key part in senescence and had been studied to greater depths in senescence, its precise role is not yet clearly understood.

Second to ethylene, abscisic acid (ABA) can be a potent senescence promoter (Coloquhoun and Hillman, 1972; Noodén, 1988c; Panavas et al., 1998). ABA and ABA-like activity have been found to rise before and during senescence in a wide variety of plant tissues, both excised and attached (Noodén, 1988c). Certain tissues that do not show an increase in ethylene levels during senescence have been found to have increased ABA levels during senescence (Panavas et al., 1998). In some tissues the effect of ABA appears to be counteracted by light. In detached tobacco leaves, kept in darkness, there was a sharp rise in ABA in the early stages of ageing, followed by a rapid decline (Evan-Chen and Itai, 1975). In leaves undergoing senescence in light, this rise was delayed by 4 days. In rose petals, the quality of light under which the plants were grown affected the time at which level of ABA rise (Garello et al., 1995). But the light-quality did not change the time of ABA rise in leaves.

The role of auxins in senescence is complicated. Generally it functions as a senescence retardant, but it can stimulate the production of ethylene, which promotes senescence in certain tissues. The endogenous IAA (indole-3-acetic acid) level in some plant tissues decreases before or during senescence (Noodén, 1988c). Application of both natural and synthetic auxins was ineffective in delaying

senescence of *Phaseolus vulgaris* leaf discs (Goldwaite and Laetsch, 1967; Stead and Moore, 1979).

Cytokinins play a fairly prominent and pervasive role as antisenescence hormone (van Staden et al., 1988). Time course studies show a decline in cytokinin levels prior to or during plant senescence (van Staden et al., 1988). Application of natural and synthetic cytokinins to plants, intact or detached plant organs frequently delays their senescence (Fletcher, 1969; Martin and Thimann, 1972; Tetley and Thimann, 1974; Lukaszewska et al., 1994; Philosphhadas et al., 1996; Franco and Han, 1997). It has been suggested that cytokinins delay senescence by inhibiting proteolysis (Kurashi, 1968; Shibaoka and Thimann, 1970).

Gibberellic acid (GA_3) is generally regarded as a plant growth promoter that delays senescence. In a wide variety of tissues, levels of gibberellic acid decline prior to or during senescence (Noodén, 1988c). In some organs, application of gibberellic acid delays senescence (Beevers 1966; Fletcher and Osborne, 1966; Goldwaite and Laetsch, 1968; Jordi et al., 1995; Franco and Han, 1997) while in others it was not effective (Goldwaite and Laetsch, 1967). Retardation of senescence by gibberellic acid was thought to be mediated through regulation of RNA synthesis (Fletcher and Osborne, 1966; Guerrero et al., 1998).

For convenience, effect of each plant growth regulator on senescence is often discussed separately. But in plants and plant organs, both intact and detached, growth rwgulators act together producing an overall outcome in promoting or delaying senescence. In a study of endogenous levels of plant hormones in tobacco leaves, Even-Chen and co-workers (1978) found a chronological sequence of three hormonal states during growth and senescence. In actively growing attached leaves cytokinin activity was high, while activities of auxin, ABA and ethylene were low. With the onset of senescence in detached leaves, there was a decrease in cytokinin activity and an increase in ABA activity, while activities of auxin and ethylene

remained low. In detached senesced leaves levels of cytokinin and ABA were low, but activities of auxin and ethylene were high. The interactive nature of these growth regulators in retarding senescence was seen when applied externally. The effect of ABA in inducing senescence is usually reversed by application of cytokinins, GA_3 and auxins. In detached tobacco leaves normal rise in ABA during senescence was inhibited by application of kinetin (Even-Chen and Itai, 1975). This was found to be due to transformation of active ABA to a bound inactive form. In the same way high concentrations of cytokinins nullified the senescence enhancing effect of low concentration of ABA in leaf discs of three plant species (Back and Richmond, 1971). In those leaves gibberellic acid was less effective than cytokinins against ABA action. Inter-relation between ABA and auxin has been studied less extensively, yet there are indications that auxin (NAA) can diminish the rise in ABA during senescence of detached tobacco leaves (Even-Chen et al., 1978).

1.1.1.8 Changes in Reactive Oxygen Species and Free Radicals

Scenescence of plant organs are accompanied by substantial increases in hydrogen peroxide and other active oxygen species such as superoxides and hydroxyl free radicals, and organic free radicals such as peroxyl and alkoxy groups, andhydrolytic products of polyunsaturated fatty acids such as hrdroperoxides (Lesham et al., 1986; Panavas and Rubinstein, 1998). Free radicals such as superoxide are liberated from unsaturated fatty acids and other cellular constituents as a result of photo-oxidation. In plants they cause post-harvest fruit spoilage, leaf senescence and accelerated wilting of cut flowers by damaging cell membranes, nucleic acids and enzymes. They also promote conversion of the ethylene precursor ACC to ethylene.

Under normal conditions, plant cells are equipped with defensive mechanisms against free radicals. Enzymes such as superoxide dismutase and catalase neutralise or decompose these toxic forms of oxygen. Endogenous antioxidants such as

ascorbic acid (vitamin C), β carotene (vitamin A) and α-tocopherol (vitamin E) are attacked and oxidised by free radicals to other harmless compounds. In senescing plant organs these protective mechanisms are found to be less active resulting in accumulation of free radicals to lethal levels (Droillard and Pauline, 1990; Panavas and Rubinstein, 1998).

1.1.2 Factors Affecting Senescence

Factors that cause or interact with the physiological changes associated with senescence have an effect on senescence of plants and plant parts. Some of these have been discussed in section 1.1.1. These include protein and RNA synthesis inhibitors, natural and synthetic plant growth regulators and their precursors, inhibitors of their synthesis and action, antioxidants, sugars, etc. In addition to these, there are some other factors that affect senescence, which will be discussed in this section. These include factors that affect senescence under natural and artificially induced conditions.

Effect of light on longevity of plants and leaves is well documented. Light affects photosynthesis in green tissues and also stomatal opening. In dark conditions lack of photosynthesis may cause nutrient deprivation leading to senescence of leaves. Light is also known have an effect on chlorophyll. In leaves light delays loss of chlorophyll and other characteristics of senescence (Goldwaite and Laetsch, 1967). Dark conditions may lead to yellowing of leaves due to breakdown of chlorophyll. Flower longevity is also affected by light (Havely and Mayak, 1979). The longevity of cut rose flowers was longer when plants were previously grown under high pressure sodium lamps than under metal halide lamps (Garello et al., 1995). It is thought that influence of light on flower longevity is due to its effect on photosynthesis and availability of carbohydrates to developing flower (Halevy and Mayak, 1979). In the same way it has been accepted that growing plants at high

temperatures causes reduced carbohydrate levels which adversely affect flower longevity (Havely and Mayak, 1979). Storage temperatures during transport of cut flowers also had an effect on their longevity (Maxie et al., 1973).

Calcium is employed by horticulturists to prolong shelf and storage life of fruits and to increase longevity of cut flowers (Lesham et al., 1986) although it appears to have the opposite effect under some instances. In association with increasing longevity of plant organs calcium application causes a marked decrease in ethylene production. Calcium is also linked to inhibition of oxidative metabolism to a certain degree. Calcium ions are also found to have effects on microviscocity of plant membranes (Borochov et al., 1978). Apart from calcium there are some other minerals that are known to affect vase life of cut flowers (Halevy and Mayak, 1981).

Certain amino acids seem to affect senescence symptoms in plant organs. One such amino acid is serine which increases the synthesis of senescence-associated proteases in leaves (Shibaoka and Thimann, 1970; Martin and Thimann, 1972). On the other hand arginine and threonine inhibited serine-induced senescence symptoms (Shibaoka and Thimann, 1970). In *Sandersonia* flowers aromatic amino acids reversed the glyphosate promoted vase life extension (Eason et al., 2000a).

In some flowers, pollination and damage to stigma or style has a dramatic effect in accelerating petal wilting and senescence (Gilissen, 1976; Nichols et al., 1983; Whitehead et al., 1984a; Piskornik, 1986; Lovell et al., 1987). Most of these studies have been carried out using *Petunia hybrida* flowers, which show an increased production of ethylene during senescence. It appears that a wilting factor is produced upon pollination or wounding of the stigma, which travels through the style rapidly resulting an immediate increase in ethylene production (Deurenberg, 1976, 1977a and b; Gilissen and Hoekstra, 1984).

Germicides have also been known to increase vase life of flowers. Application of 8-hydroxyquinoline citrate (250 mg/L), n-alkyl dimethyl ethylbenzyl ammonium chloride (50 mg/L), sodium dichloroisocyanuric acid (50 mg/L) to *Gloriosa rothschildiana* prolonged flower longevity by 12% (Jones and Truett, 1992). This increase in vase life was achieved by maintaining the fresh weight of flowers and solution uptake. Similarly 8-hydroxyquinoline citrate (200 mg/L) improved water uptake and leaf moisture retention of flowering stem cuttings of *Leptospermum scoparium* Forst., and prolonged the vase life of the flowers (Burge et al., 1996).

The pH of the vase solution also has an effect on longevity of cut flowers. Acidic solutions appear to improve vase life of flowers of *Gloriosa rothschildiana* when compared to neutral solutions (Jones and Truett, 1992). Acidity of the solution is thought to improve water uptake of cut flowers by reducing microbial growth and also by retarding stem blockage (Havely and Mayak, 1981).

Factors that cause diseases and stress conditions to plants and plant parts may also induce senescence-like responses. Sensitivity of a plant or plant part to these factors, when applied exogenously, largely depend on the nature and stage of development of the plant or plant part, time of application, concentration applied, and other factors that may be present under the condition of study. The sensitivity of plant parts to externally added growth regulators is a very good example of this (Woodson et al., 1985; Hoekstra and Weges, 1986; Woltering, 1987; Warman and Solomos, 1988). In *Hibiscus roas-sinensis* L., application of ACC, a precursor of ethylene, to immature petals did not induce senescence. In contrast application of the same compound to mature petals accelerated senescence (Woodson et al., 1985).

1.1.3 Flower Senescence

Floral senescence should occur when the expected fitness gain per unit of floral maintenance investment diminishes to the point where it becomes more profitable to construct a new flower than to maintain an existing one (Ashman and Schoen, 1994). Flower is a complex organ, mainly consisting of a green calyx, corolla, a gynoecium and an androecium. Each of these structures is complex on its own right and differs from each other both in structure and physiology. The interrelationship between these organs may determine the rate of senescence. For example, as in many flowers, acceleration of corolla senescence occurs after pollination.

Due to the structural complexicity of the flower, most studies on flower senescence are usually restricted to different organs of the flower. Since petals are the organs that make flowers economically valuable many studies have been carried out on petal senescence. The shorter life span of petals compared to the other plant organs makes petals an excellent model system for the study of fundamental senescence processes. In addition to the petals, changes taking place in the gynoecium during senescence have also attracted the interest of researchers due to its possible involvement in inducing senescence after pollination.

Similar to any other plant organ senescence, flowers show a variety of patterns. In 1928, Pfeiffer recognised three types of petal senescence: (1) petal fall without previous wilting or with concomitant wilting; (2) petal wilting and late fall or no fall; (3) greening of petals without fall. After studying flowers of different genera in many families Woltering and van Doorn (1988) categorised petal senescence based on sensitivity to ethylene: (a) wilting apparently mediated by ethylene, (b) wilting apparently not mediated by ethylene and (c) abscission that is apparently mediated by ethylene. Flowers whose petal wilting is mediated by ethylene are called ethylene-sensitive flowers, while flowers whose petal wilting is not mediated by ethylene are called ethylene-insensitive flowers. In ethylene-sensitive

flowers, senescence is usually accompanied by a respiratory peak and an ethylene production peak. Exposure of these flowers to exogenous ethylene generally hastens the onset of senescence and the development of typical aging symptoms (Suttle and Kende, 1980; Woodson et al., 1985). On the other hand, in ethylene-insensitive flowers there is no peak in ethylene production during senescence. Although some ethylene-insenestive flowers do not show a climacteric respiratory peak during senescence, there are some ethylene-insensitive flowers that show a transient respiratory peak during senescence (Lukaszewski and Reid, 1989; Bieleski and Reid, 1992; Serek et al., 1994). Also in these flowers exogenous ethylene has no effect on senescence. Ethylene-sensitive flowers include carnation (Whitehead et al., 1984a and b; Woodson, 1987), hibiscus (Woodson et al., 1985), orchids (Woltering and van Doorn, 1988) *Ipomoea*, *Tradescantia* (Suttle and Kende, 1978) and *Petunia*. Examples of ethylene-insensitive flowers are *Zinnia* (Stimart et al., 1983) *Chrysanthemum* (Williams et al., 1995), *Sandersonia* (Eason and de Vré, 1995), daylily (Lukaszewski and Reid, 1989; Lay-Yee et al., 1992), *Gladiolus* (Woltering and van Doorn, 1988), *Narcissus* (Woltering and van Doorn, 1988), tulip (Jones et al., 1994) and *Gloriosa* (Jones and Truett, 1992).

After carrying out an extensive study among monocotyledons McKenzie and Lovell (1992) distinguished three broad types of flower senescence: abscission of flowers (thus floral parts are persistent); abscission of floral parts (perianth, style and/or stamens) only; and withering of flowers and constituent floral parts (i.e. both persistent).

Like with any other plant organs, petal senescence is also associated with many ultrastructural, biochemical and biophysical changes (Mayak and Halevy, 1980). Although most of these changes are discussed above in section 1.1.1, it is worthwhile summarizing those changes again here. Changes taking place during flower senescence are: (1) loss of fresh weight, drying and shrivelling, (2) invagination of the tonoplast, indicating autophagic activity of the vacuole,

decrease in the membrane fluidity and increased membrane permeability, and loss of phospholipids from membranes, (3) increased respiration and hydrolysis of cell components, (4) a sharp increase in activity of some hydrolytic enzymes, for example, RNAase, DNAase, and hydrolases of cell wall polysaccharides, (5) a drop in the level of macromolecular components, for example, starch, polysaccharides, proteins and nucleic acids, (6) transportation of sugars, ions and breakdown products of the macromolecules out of the petals to other parts of the plants or to the developing gynoecium, (7) an increase in the pH of the vacuole and (8) change in pigmentation.

1.1.4 Senescence Mechanisms

Traditionally causes of senescence are categorised into two groups, nutrient deficiencies (e.g. starvation) and genetic programming. Since senescence is often associated with the end of the useful period of an organ or plant (e.g end of reproduction) and nutrients are often (but not always) redistributed from senescing organs to other developing organs, nutrient starvation has been invoked as the cause of senescence. Although there is evidence that nutrient drain and diversion play a role in senescence, it appears that these are not the primary causes of this phenomenon (Lesham et al., 1986). Senescence is more likely to be an internally programmed process, because it is specific and orderly in terms of when, where and how it occurs (Noodén et al., 1997). It is speculated that senescence takes place in a stepwise manner (Figure 1.1). It is controlled by internal and external signals, and it can be delayed or accelerated by altering these signals (Noodén, 1988a). The internal programming of senescence implies that it is under genetic control. However, senescence is not caused by turning genes off completely (Nooden, 1988a). Thus the process might begin with changes in gene expression, causing alterations in RNA and protein synthesis. Shutting off synthesis of certain RNAs and proteins may be required to prevent regeneration of cellular components that

are broken down during senescence (Noodén et al., 1997), but the selective activation of the certain mRNAs and proteins seems more likely the initiation of the active processes leading to senescence.

There is strong evidence suggesting that the expression of genes have some role in causing senescence in plants and plant organs. Slater and co-workers (1985) have found 146 ripening related cDNA clones from tomato fruit. Oh and co-workers (1996) characterised the structure and expression of a senescence-associated gene from *Arabidopsis thaliana.* During senescence, expressions of many genes are down regulated (Woodson, 1987; Wang et al., 1993). However, many genes that are upregulated during senescence have also been identified in plants from leaves and flowers (Woodson, 1987; Lawton et al., 1989; Valpuesta et al., 1995; Guerrero et al., 1998; Panavas et al., 1999; Eason et al., 2000b). In ripening avocado fruits, three mRNAs increased dramatically with the climacteric rise in respiration and ethylene production (Christoffersen et al., 1982). Evidence for the presence of a genetic basis in causing senescence has also come from ripening-impaired tomato plants (Brady et al., 1983; DellaPenna et al., 1987; Sheehy et al., 1988; John et al., 1995).

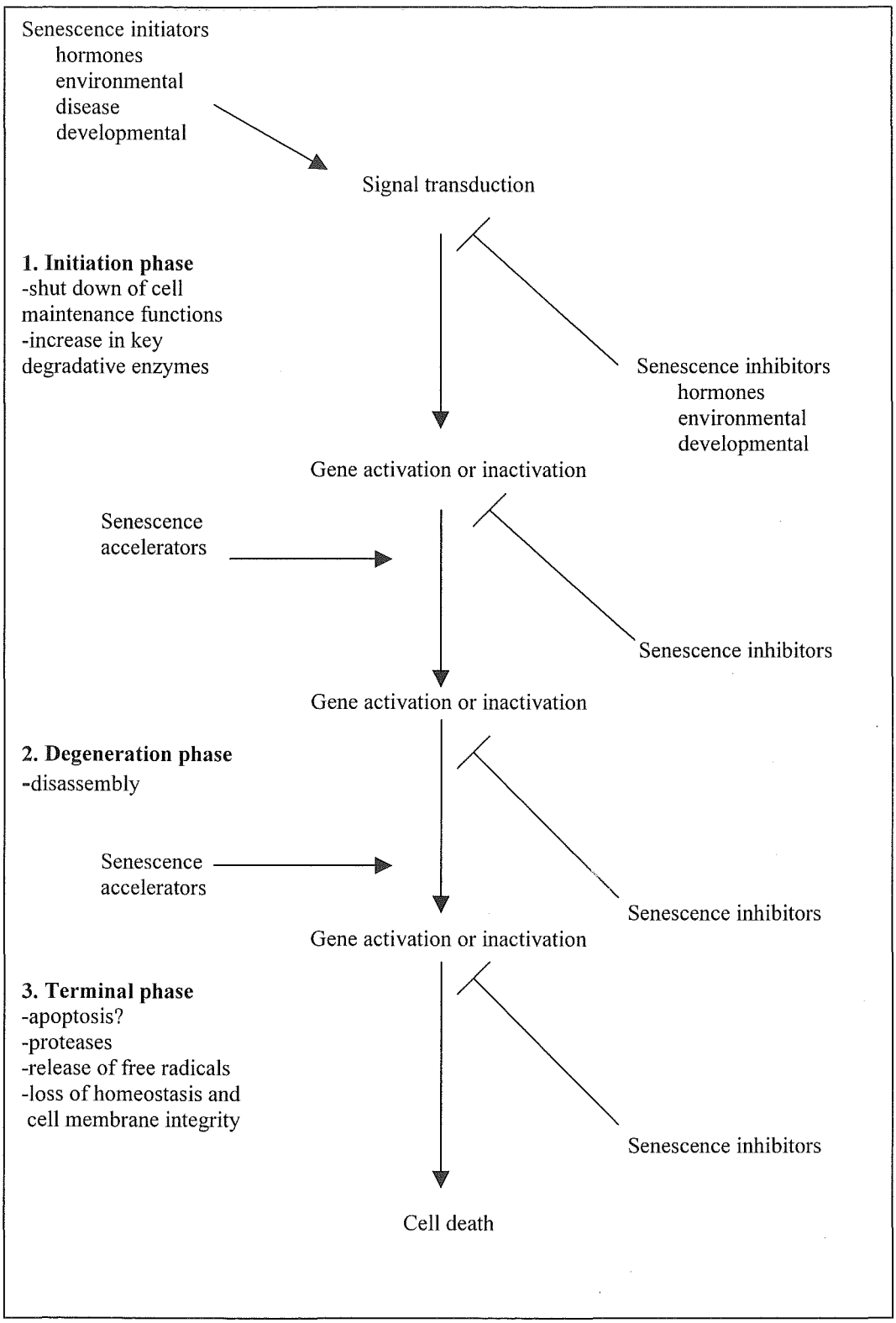


Figure 1.1: Proposed outline of the central pathways for leaf senescence (adapted from Noodén et al., 1997).

1.2 PROTEIN DEGRADATION IN PLANTS

Like in all other organisms, proteins in plants are continuously degraded and replaced. Although this phenomenon appears to be a wasteful process, it is essential for plants to re-utilise amino acids, to change their protein content during development, and to adapt to new environmental conditions (Vierstra, 1993). One of the main functions of protein degradation is to break down abnormal proteins that are continuously arising due to various causes such as mutations, biosynthetic errors, spontaneous denaturation, damages caused by free radicals, environmental purtabations, and diseases. This is essential in order to avoid accumulation of these proteins to toxic levels. Other possible functions of proteolysis include the following (Viestra, 1993: 1996; Coux and Piechaczyk, 2000): (1) the stoichiometric accumulation of enzyme subunits and maintenance of the correct enzyme/cofactor ratios (Vierstra, 1993), (2) conferring short half-lives of enzymes where levels must be precisely controlled depending on the nutritional or developmental state, (3) generation of peptides that act as hormones, antigens, or other effectors, and for the removal of signal and transit peptides following transport of proteins into the endoplasmic reticulum, mitochondria and chloroplasts (Vierstra, 1993), (4) supplying the amino acids necessary for maintaining cellular homeostasis and growth, (5) during programmed cell death where proteins from single cells, organs and tissues are mobilised to other organs of the plant and (6) improving crop plants via genetic engineering for the production of economically important proteins. Since, plant cells recognise and degrade proteins produced by foreign genes, inactivation or redesigning of such proteases will allow accumulation of the desired gene products adequately.

Even though protein breakdown in plants has been studied for almost 30 years, it is only within the last decade that we have begun to understand why and how protein degradation occurs (Vierstra, 1993). These findings indicate that protein degradation involves several distinct pathways with each cellular compartment having its own system (Klerk and van Loon, 1997).

One of the well-studied pathways is ubiquitin-dependent proteolytic pathway in which cytoplasmic and nuclear plant proteins are degraded. In this pathway, ubiquitin, a polypeptide containing 76 amino acid residues becomes covalently attached to proteins that are soon to be degraded (Viestra, 1996; Coux and Piechaczyk, 2000; Kierszenbaum, 2000). This involves ATP and the sequential action of three enzymes, E1s, E2s, and E3s, each of which constitutes a family of isozymes. After ligation, the protein molecule may be subjected to one of the following three fates. It can be degraded by an ATP-dependent protease, identified as the 26S proteasome that is specific for ubiquitin-protein conjugates, releasing ubiquitin intact to be reused. The 26S proteasome preferentially breaks substrates with multi-ubiquitin chains, thus providing a mechanism to target proteins based on the amount of modification by ubiquitin (Kierszenbaum, 2000). It is also possible that (1) the protein-ubiquitin conjugate can be disassembled by a class of ubiquitin-protein hydrolases that cleave only the γ-amino isopeptide bond ubiquitin and the target protein, allowing the target protein to escape degradation (2) the conjugate may exist stably in the cell. Mostly these long-lived conjugates are mono-ubiquinated, that have been escaped the 26S proteasomes.

Like lysosomes in animal cells, vacuoles in plant cells are also thought to be involved in protein degradation (Canut, et al., 1985; Moriyasu and Tazawa, 1998). These are acidic compartments containing a wide variety of hydrolytic enzymes including proteases. However, the mechanisms by which the proteins are broken down in vacuoles are unclear.

A protein body, a specialised form of a vacuole in seeds is a major site of protein degradation during seed germination (Natacha-B, et al., 1998). Proteins in the protein body are deposited during seed formation. During seed germination, ATP-independent proteases are imported into the protein body. In cereal seed aleurone layers, gibberellic acid induces the *de novo* synthesis of these proteolytic activities (Mikonen, et al., 1996).

Chloroplasts play an important role in degrading proteins in photosynthetic tissues using their own internal proteases (Spetea, et al., 1999). Some of these enzymes require ATP (Georgakopoulous and Argyroudi, 1997). One of the most important and well-studied chloroplast protease is the homolog of the ATP-dependent ClpA/P protease from *E. coli* (Ostersetzer, et al., 1996). Other organelles such as mitochondria, microbodies and endoplasmic reticulum have proteolytic pathways that are involved in the maturation and function of each compartment (Viestra, 1996).

1.2.1 Proteolytic Enzymes

The role of proteolytic enzymes in all life forms can be viewed as part of an overall process of protein turnover. After synthesis, proteins begin their journey toward degradation, whether they are intracellular or extracellular, with half-lives of a few minutes, hours, days or perhaps months or years. Inevitably, proteins are broken down and reutilised, either by the organism that produced them or by other organisms, to support new life processes. During this journey many proteins undergo proteolytic modifications, or processing steps, preceding their final breakdown (Ryan and Walker-Simmons, 1981).

Proteins are usually hydrolysed to amino acids by sequential co-orporation of three types of enzymes. First, the long polypeptide chains are split to short polypeptides by endopeptidases, which hydrolyse peptide bonds in the middle of type chains with varying degrees of specificity. Next, exopeptidases sequentially liberate amino acid residues or dipeptide units from either end of the peptide chains. The enzymes acting at the N-terminus are called aminopeptidases and dipeptidyl aminopepetidases while those acting at the C-terminus are called carboxypeptidases and dipeptidyl carboxypeptidases. Finally, the remaining dipeptides are hydrolysed by dipeptidases (Mikola and Mikola, 1986). All these enzymes are collectively designated as

peptidases while the terms proteinases and proteases are equivalent to the endopeptidases (Kenny, 1999). A representative diagram of the main classes of peptidases is shown in Figure 1.2. According to the 'enzyme commission' (E.C.) classification (International Union of Biochemistry and Molecular Biology, 1992) peptidases are designated as E.C.3.4. The main types of peptidases are defined by a third numeral (Table 1.1).

1.2.1.1. Endopeptidases

It is rare to find an endopeptidase that is specific for a bond involving a single type of side chain and, therefore there is no classification related to specificity (Kenny, 1999). Hence according to catalytic mechanism, endopeptidases are divided into four main groups: serine endopeptidases with a serine residue in the active centre, cysteine endopeptidases having amino acid cysteine in the active centre, aspartate endopeptidases that depends on two aspartic acid residues for their catalytic activity and metallo-endopeptidases that use a metal ion (usually zinc) in the catalytic mechanism (Barret, 1994).

1.2.1.2 Carboxypeptidases

Carboxypeptidases are common components of many plant genera and probably present in all higher plants, monocots, dicots and gymnosperms, and at least in most plant tissues (Mikola and Mikola, 1986). They are classified according to the class of active site, three types being recognised: serine, metallo and cysteine (Kenny, 1999). Most of them are classified as Zn-metallopeptidases (Kenny, 1999), although metallo-carboxypeptidases appear to be rare in plants (Ryan and Walker-Simmons, 1981).

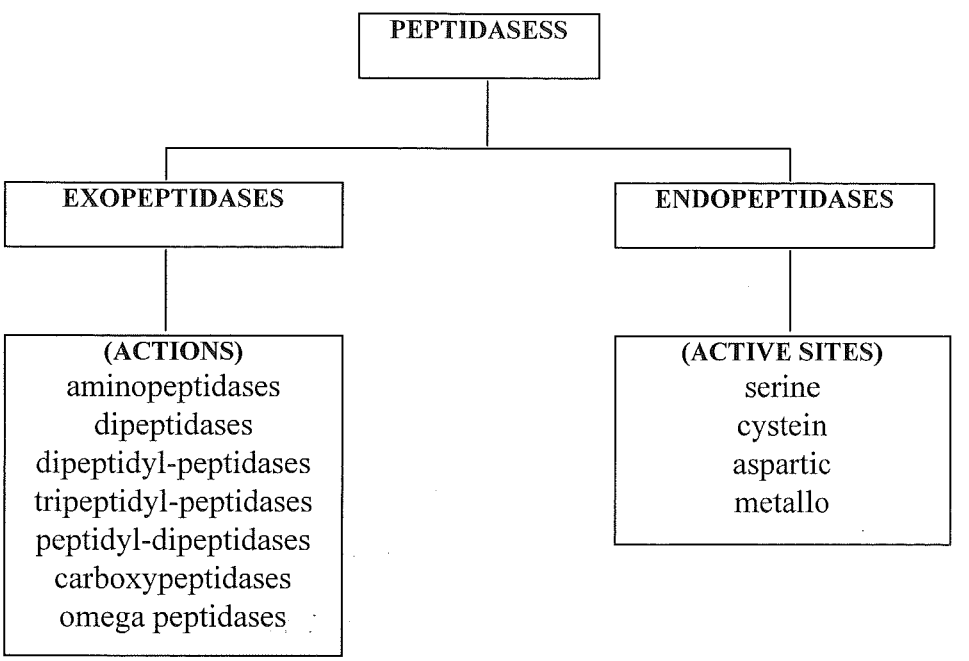


Figure 1.2: The main classes of proteolytic enzymes (Kenny, 1999).

Table 1.1: Types of peptidase defined in the enzyme nomenclature list of the International Union of Biochemistry and Molecular Biology (1992).

E.C. number	Peptidase type	Action
Exopeptidases		
3.4.11.-	Aminopeptidase	N-terminal residue released
3.4.13.-	Dipeptidase	Acts only on dipeptides
3.4.14.-	Dipeptidyl peptidase	N-terminal dipeptide released
	Tripeptidyl peptidase	N-terminal tripeptide released
3.4.15.-	Peptidyl dipeptidase	C-terminal dipeptide released
3.4.16.-	Carboxypeptidase (serine)	C-terminal residue released
3.4.17.-	Carboxypeptidase (metallo)	C-terminal residue released
3.4.18.-	Carboxypeptidase (cysteine)	C-terminal residue released
3.4.19.-	Omega peptidase	Releases modified residues from N- or C-termini
Endopeptidases		
3.4.21.-	Serine endopeptidase	
3.4.22.-	Cysteine endopeptidase	
3.4.23.-	Aspartic endopeptidase	
3.4.24.-	Metallo-endopeptidase	
3.4.99.-	Endopeptidase of unknown catalytic mechanism	

1.2.1.3 Aminopeptidases

Aminopeptidases are ubiquitous enzymes, and a wide variety of aminopeptidase activity had been found in animal, plant and other eukaryotic cells. Traditionally, aminopeptidases were classified based on the substrate used in the activity assay (Walling and Gu, 1996). There is another classification system of aminopeptidase based on the optimum pH for enzymatic activity and substrate specificity (Mikola and Mikola, 1986). According to this classification, there are two major groups of plant aminopeptidases, the neutral and alkaline aminopeptidases. Most of the aminopeptidases, characterised so far, belong to neutral aminopeptidases (Kolegmainen and Mikola, 1971; Elleman, 1974; Palaviccini et al., 1981; Tazaki and Ishikura, 1984; Waters and Dalling, 1984). These are thermolabile, monomeric enzymes of approximately 60-90 kDa with a neutral pH optimum. They are insensitive to metal chelators but are strongly inhibited by heavy metals and SH-reagents (Ryan and Walker-Simmons, 1981). Neutral aminopeptidases are subdivided into groups according to their substrate specificity. The first group ("Phe" group) preferentially hydrolyse β-naphthylamides conjugated to aromatic (Phe, Tyr, Trp) or bulky hydrophobic (Leu, Met) amino acids. These enzymes need sulfhydryl groups for their activity or structural integrity (Walling and Gu, 1996) and are monomers with molecular masses between 56 and 76 kDa. The second group of neutral aminopeptidases preferentially hydrolyse substrates with N-terminal alanine or leucine residues ("Ala-Leu" aminopeptidases). Most of these aminopeptidases are metalloenzymes with a wide range of sizes varying from 14 to 390 kDa. The third group of neutral aminopeptidases in plants are the proline aminopeptidases or iminopeptidases. These aminopeptidases have a high molecular weight (168-440 kDa) and are inhibited by the sulfhydryl group inhibitor pHMB, but not by chelating agents (Mikola and Mikola, 1986).

Alkaline aminopeptidase activities have been detected in a wide variety of plants (Sopanen and Mikola, 1975; Mikkonen and Mikola, 1986; Mikkonen, 1992). Most alkaline aminopeptidases hydrolyse peptide, β-naphthylamide and ρ-nitroanilide

substrates (Walling and Gu, 1996). Alkaline aminopeptidases include enzymes with a wide range of properties. Many alkaline aminopeptidases are sensitive to sulfhydryl group inhibitors such as pCMB and pHMB. Some are metalloenzymes.

1.2.1.3.1 Leucine aminopeptidase (LAP)

The enzymes that remove Leu and other hydrophobic residues from peptide substrate analogs preferentially, but not exclusively are called LAPs (Taylor, 1993).

LAPs are the most characterised (Table 1.2) alkaline aminopeptidases from plants, which form a discrete group. These aminopeptidases show a remarkable similarity to the LAPs from mammals and prokaryotic organisms (Sopanen and Mikola, 1975; Walling and Gu, 1996). These are large (250-330 kDa), homohexameric, metallo enzymes with subunit mass of about 55 kDa. They have an alkaline pH optima and are thermostable. Their activity is inhibited by chelating agents, amastatin and bestatin but are usually unaffected by SH-reagents (Prescott and Wilkes, 1966; Sanderink et al., 1988; Kaga et al., 1998).

The first plant *lap* cDNA clone was isolated from *Arabidopsis thaliana* by screening an expression library using polyclonal antibodies against purified plasma membrane proteins (Bartling and Weiler, 1992). Immunoblot studies showed that the enzyme is present in all developmental stages and in all organs examined and is not induced in response to mechanical wounding or exogenous growth regulators (Bartling and Nosek, 1994). In contrast, tomato and potato *lap* cDNAs encode mRNAs which are induced by mechanical wounding or wounding signals such as ABA and jasmonic acid (Hildmann et al., 1992; Bartling and Nosek, 1994; Herbers et al., 1994). In tomato *lap* mRNAs are rapidly induced locally and systemically in response to wounding, insect infestation and *Psudomonas syringae* pv tomato (Bartling and Weiler, 1992; Pautot et al., 1993). On the other hand, potato *lap* mRNAs are not

induced systemically or in response to fungal or bacterial invasion (Hildmann et al., 1992; Herbers et al., 1994).

1.2.2 Developmental Regulation of Proteolytic Enzymes

Development is the process by which a programme of selective gene expression, operating on a constant pool of genetic information produces a complex adult organism from a single fertilised cell. A major aspect of this process is tissue differentiation, i.e. the generation of the many cell types, each with specific physical and metabolic characteristics, which are essential to the diverse and integrated functions of the adult organism. In bringing up these changes, enzymes including proteolytic enzymes play an indispensable role. It is very clear that the activities of these enzymes are regulated during the process of development of organs and organisms. Such developmental regulation by proteolytic enzymes can be seen from the seed development to the senescence of plants and plant organs. Changes in all three proteolytic enzymes occur during seed formation (Kruger and Preston, 1978; Degan et al, 1994) and germination (Chrispeels and Boulter, 1975; Doi et al., 1980; Leung and Bewley, 1983; Mikola, 1983; Isola and Franzonir, 1996) in both monocots and dicots. The main function of these enzymes, during seed germination, may be to supply free amino acids to developing seedlings. After germination the levels of proteolytic enzymes continue to be changing during seedling growth. Such changes have been observed in young developing leaves, roots and coleoptiles (Andrews et al., 1979; Waters and Dalling, 1979; Doi et al., 1980).

Table 1.2: Characters of LAPs from plant sources[A].

Plant source	Mol.Wt (kDa)[B]	substrates[C]	PH optimum	Inhibitors[D]	Activators	Mechanism	Reference
Hordeum vulgarae (barley)	260	Leu-Tyr; Leu-Gly, Leu-Gly-Gly, Met-Leu-Gly	8.5-10.5	Need sulfhydryl compounds and Mn^{++} or Mg^{++} for full functionality.			Sopanen and Mikola, 1975.
Arabidopsis thaliana	320 (55)	Leu-p-NA	8.5	Zn^{++}, Bestatin, 1,10-phenanthroline, pCMB	Mn^{++}, Mg^{++}	Metallo enzyme	Bartling and Weiler, 1992.
Phaseolus vulgaris L. Cl. Processor (kidney bean)	360 (58 & 60)	Leu-Gly-Gly, Leu-Gly, Ala-Ala-Pro	9.0	Bestatin, Zn^{++}, o-phenanthroline	Mg^{++}	Metallo enzyme	Mikkonen and Mikola, 1986; Mikkonen, 1992.
Solanum tuberosum L. (potato)	n.d. (54)	Leu-p-NA, Arg-p-NA, Met-p-NA	10	Bestatin, Zn^{++}, EDTA, PMSF	Mn^{++}	Metallo enzyme	Herbers, et al., 1994.
Lycopersicon esculentum (tomato)	>327 (55)	Leu-p-NA, Leu-β-NA, leucinamide	n.d	n.d.	-	-	Walling and Gu, 1996.

A: Through out the table, n.d.:not determined; -: not mentioned.

B: Molecular weight of native enzyme is expressed in kDa. The sizes of the LAP subunits are indicated in parentheses.

C: p-NA: ρ-nitroanilide; -β-NA: -β-naphthylamide.

Studies on developmental regulation of proteolytic enzymes in leaves have been carried out using intact and detached leaves both from mature plants and from seedlings. Two endopeptidases Azocollase A and B, that act on azocoll, have been characterised from soybean leaves (Ragster and Chrispeels, 1979). There was a gradual increase in the total Azocollase activity during leaf maturation, but this declined during senescence. In young leaves the predominant enzyme of the two was Azocollase B. During leaf maturation there was a dramatic increase in Azocollase A while Azocollase B increased slowly.

In barley, although aminopeptidase activity decreases as leaves undergo senescence, no new aminopeptidase isoforms appeared during senescence and none disappeared (Thayer et al., 1988). In these leaves the highest aminopeptidase activity was found in the young developing leaves, and as the leaves reached maturity the enzyme activity declined. This was observed one day before degradation of 85% of the soluble leaf protein.

In oat leaves, senescence was associated with an increase in two proteases that act on haemoglobin, while total leaf protein was decreasing (Martin and Thimann, 1972). Presence of L-serine increased the amount of these two enzymes, but presence of cycloheximide and kinetin greatly decreased them. Therefore kinetin may delay senescence by suppressing protease formation (Martin and Thimann, 1972). In corn, foliar senescence symptoms are paralleled by decreases in exopeptidase activities (aminopeptidase and carboxypeptidase), protein and chlorophyll and an increase in endopeptidase (caseolytic) activity (Feller et al., 1977). There was an increase in three forms of aminopeptidases in *Eunomyces* leaves undergoing the process of senescence in autumn (Tazaki and Ishikura, 1983). In wheat leaves, proteinases that increase in activity during senescence had a high affinity towards ribulose biphosphate carboxylase enzyme (Wittenbach, 1978 and 1979).

In corn leaves, aminopeptidase and carboxypeptidase activities increase with the onset of the visible reproductive phase while leaf protein content and chlorophyll content are still high and constant (Feller et al., 1977). They decrease concurrently with loss of protein and chlorophyll. In contrast, caseolytic activity increases. This suggests a major role for the caseolytic enzymes in initiating rapid hydrolysis of leaf proteins. Exopeptidases may have a role in the mobilisation of leaf proteins during grain development.

Although it is highly likely that proteolytic enzymes play an important role during flower development and senescence, there are only few studies carried out on this subject. In studying hydrolysis of seven peptide substrates by petal extracts of tulip, Sopanen and Carfanten (1976) showed that there was a slight increase in carboxypeptidase and aminopeptidase activity per flower during bud development. These activities increased to an optimum at the beginning of withering and remained high during senescence. In daylily, there were no detectable levels of endopeptidase activity during bud development when gelatin and azoalbumin was used as the substrate (Stephenson and Rubeinstein, 1998). The endopeptidase activity per flower increased after the flower is fully open and reached an optimum 12 hr later. In potato flowers LAP activity based on the total protein content remained more or less constant during development from bud to open flowers (Herbers et al., 1994).

Changes in proteinases are seen during development of ovary into a fruit. In unpollinated pea ovaries, endopeptidases have been found to play a key role during senescence (Carrasco and Carbonell, 1990) while exopeptidases were important in gibberellic acid induced fruit development. Similarly in tomato, increase in carboxypeptidase activity was seen during fruit ripening (Matoba and Doi, 1974; Mehta et al., 1996).

1.2.3 Inhibitors of Proteolytic Enzymes

Inhibitors of proteolytic enzymes are molecules that bind at or near the active site to produce a decrease in catalytic activity (Knight, 1999). They can be divided into two groups as synthetic (or exogenous) inhibitors and endogenous inhibitors.

1.2.3.1 Synthetic Inhibitors

Synthetic inhibitors may be subdivided into two major classes, namely transition-state enzyme inhibitors and suicide enzyme inactivators (Rappay, 1989). Suicide inactivators are substrates that are subjected to normal catalysis by the target enzyme resulting in a potent intermediate, which reacts irreversibly with the parent enzyme. The transition-state enzyme inhibitors are active site directed molecules, which mimic the structure of the natural substrate of the proteinase inhibited by it.

To fully understand the molecular and/or physiological functions of a proteolytic enzyme, it is important to know how to inhibit its activity. Presently, there are four known catalytic mechanisms by which an enzyme can cleave a peptide bond. These are named after the chemical group most directly involved in bond cleavage and comprise the serine proteases, the cysteine proteases, the aspartic proteases and metalloproteases. There are class specific inhibitors for each of these enzyme classes, thus when characterising enzymes of unknown mechanism, the obvious strategy is to test the effect of inhibitors from each class. These inhibitors are known to alter the amino acids that are vital for the enzyme activity. These include artificially synthesised compounds as well as compounds purified from organisms.

Serine protease inhibitor diisopropyl fluorophosphate (DFP) is an organophosphate compound that acylates a single serine residue at the active site irreversibly (Kenny, 1999). Other serine protease inhibitors include phenylmethylsulfonylfluoride (PMSF), 3,4-dichloroisocoumarin (DCI), N-tosylamidophenylalanine chloromethyl

ketone (TPCK also named L-1-chloro-3-tosylamido-4-phenyl-2-butanone) and N-tosyl-lysine chloromethyl ketone (TLCK) (Wolz, 1999). In the catalytic mechanism of cysteine peptidases the thiol group of a single cysteine residue plays an essential role. Generally, cysteine protease enzymes are active only when the active site cysteine is in the reduced form (Wolz, 1999). This thiol group in the cysteine residue is highly susceptible to oxidation which can be inhibited by iodoacetamide (IAM), iodoaetic acid, N-ethyl-maleimide (NEM), E64 (L-trans-epoxysuccinyl-leucylamido-(4-guanidino)butane), amino acid diazomethylketones and peptide diazomethylketones. An indication of the aspartic type proteolytic enzymes is that they are sometimes optically active at low pH (Wolz, 1999) and have a low pH optimum (Kenny, 1999). The inhibitor considered to be most clearly diagnostic for an aspartic protease is pepstatin. Other aspartic enzyme inhibitors are diazomethylketones and EPNP ((1,2-epoxy-3-) ρ-nitrophenoxy)-propane). The metallopeptidases are usually recognised by their susceptibility to inhibition by chelating agents such as EDTA, 1,10-phenanthroline and amino acid hydroxamates. These inhibitors usually interact with catalytically essential metal ion. A disadvantage of using EDTA in identifying metallopeptidases is that it chelates with Ca^{++} and therefore inhibits Ca^{++} dependent serine- and cysteine proteases also (Wolz, 1999). Characteristics and mechanisms of inactivators of some of the synthetic inhibitors are shown in Table 1.3.

Table 1.3: Characteristics of some of the proteolytic enzyme inactivators (adapted from Walz, 1999).

PMSF	
Mechanism of inactivation	Irreversible, covalent modification of the active site serine.
Specificity	General inhibitor of all serine proteases. Does not inhibit serine protease zymogens. May also inhibit some cysteine proteases, but in that case, inhibition can be reversed by the addition of thiol reagents such as 2-mercaptoethanol.
Solubility	Soluble to > 10 mg/mL (57.4 mM) in alcohols (ethanol, methanol, propanol); not appreciably soluble in water.
Stability	Unstable in aqueous solution, but stable in alcohol for at least 9 months at 25°C.
IAM	
Mechanism of inactivation	Covalent alkylation of the active site cysteine residue.
Specificity	Reacts with reduced cysteine residues in any protein.
Solubility	Soluble in water or alcohol.
Stability	Prepare fresh.
Pepstatin	
Mechanism of inactivation	Tight, but reversible binding. It is a transition state analog.
Specificity	Specific for most known aspartic proteases.
Solubility	Soluble in methanol to 1 mg/mL. Slowly (12 hr at room temperature) soluble in ethanol to 1 mg/mL. Soluble in 6 M acetic acid to 0.3 mg/mL.
Stability	Stable at least one week at 4°C.
EDTA	
Mechanism of inactivation	Chelates free metal in solution (usually).
Specificity	Can inhibit all metalloproteases and other metalloenzymes. Forms complexes with Ca^{++}, Cu^{++}, Fe^{++}, Pb^{++}, Mg^{++}, Mn^{++}, Ni^{++} and Zn^{++}. Can inhibit Ca^{++} dependent serine- and cysteine proteases.
Solubility	Soluble in water to 500 mM at pH 8-9. The disodium salt will not dissolve unless the pH of the solution is adjusted to near pH 8 with NaOH.
Stability	Stable in solution at 4°C for at least 6 months.

1.2.3.2 Endogenous Inhibitors of Proteolytic Enzymes from Plant Sources

Proteinaceous proteinase inhibitors are widely distributed in the plant kingdom. Most of these inhibitors are present in certain storage organs such as seeds and tubers of various plants. Seeds of the family Leguminosae and of the family Solanaceae are excellent sources of proteinase inhibitors. For example, there were cysteine proteinase inhibitors in seeds of Mimosoideae tree *Adenanthera pavonina* (Silva et al., 1995), seeds of sunflower (Kouzuma et al., 1996) and sweet potato (Scott and Symes, 1996). A carboxypeptidase inhibitor have been purified and characterized from potato tubers (Hess and Ryan, 1981) while a papain inhibitor had been found from bean seeds (Santino et al., 1998). Some of these proteinase inhibitors have been purified.

These inhibitors can be diverse in number and in specificity towards various proteolytic enzymes. Besides there can be several different kinds of inhibitors present in a single tissue. For instance, six proteinase inhibitors have been purified from winter pea seeds (Ferrasson et al., 1997). Seeds of bitter gourd (*Momordica charantia*) contained two inhibitors that act on tripsin and elastase (Hamato et al., 1995). The multiplicity of plant proteinase inhibitors may partly be ascribed to the self- and mixed-association of a few monomers in each plant as well as to partial proteolysis of the inhibitors during purification, especially when the inhibitors are purified by affinity chromatography (Birk, 1976).

The physiological importance of plant proteinase inhibitors could be attributed to defence mechanisms against predatory insects. Presence of inhibitors of growth and larval gut proteases of the intestines in ornamental tobacco *Nicotiana alata* (Heath et al., 1997) suggests the possibility that these inhibitors may have evolved as a defence mechanism. In addition, induction of cysteine proteinase inhibitors by wounding or methyl jasmonate ('wound hormone') treatment on leaves of soybean (Zhao et al., 1996), induction of proteinase inhibitors in tomato and potato leaves after attack by

Colorado potato beetles and their larvae (Green and Ryan, 1972) and wounding (Thaler et al., 1996) further support this hypothesis.

1.3 MICROPROPAGATION OF PLANTS

Micropropagation is the procedure utilized for multiplying plants under aseptic conditions using small explants or cuttings. This can be accomplished by multiplication of shoots from explants of shoot tips or axillary buds, direct formation of adventitious shoots or somatic embryos on explants of tissues or organs, or by formation of shoots or somatic embryos from callus or semi-organised callus obtained from explants (Sagawa and Kunisaki, 1990).

Propagation of a plant through tissue culture proceeds through a sequence of steps: establishment of the aseptic culture, multiplication of propagule, preparation for reestablishement of plants in soil and transfer to natural environment. An additional step for selection and preparation of mother plants may be required for certain species especially in reducing contaminations.

Micropropagation has the following advantages over conventional methods of propagation (Hussey 1983; Sagawa and Kawasaki, 1990): (1) Ability to produce large number of plants of the same phenotype using only small pieces (explants) of plants; (2) Ability to produce large number of plants in a small space and within a relatively short period of time; (3) Ability to store plants in a small space; (4) Need of less space and labour for maintenance of stock plants; (5) Ability to produce pathogen free-plantlets; (6) Ability to control nutrient levels, light, temperature, and other factors with relative ease to accelerate vegetative multiplication and regeneration and to get improved phenotypes that can be altered by external conditions; (7) Need for minimal attention between subculture and no need for labour and material for watering, weeding, spraying etc; (8) Ability to propagate

even during the off season; and (9) Ability to propagate genetically improved plant varieties that are otherwise seed sterile.

There are some disadvantages associated with microprpagation compared to conventional methods (Hussey 1983; Sagawa and Kawasaki, 1990). They are: (1) Need for advanced skills and specialised equipment and facilities which may increase the production cost; (2) Propagules used in micropropagation are relatively expensive due to labour-intensive methods used; (3) To get optimum results, specific methods should be developed for each species; (4) Plantlets are initially very small; and (5) There is a high possibility of producing somaclonal variations.

1.4 DAYLILIES

1.4.1 Origin, History and Spread of Daylilies

Daylilies, like many of the bulbous plants with flamboyant flowers were thought to be native to the countries around the Mediterranean and also Southeast Asia including China, Japan and Korea. They have been cultivated in China for thousands of years mainly for their food and medicinal value. Daylily had inspired many Chinese poets and artists. They were mentioned in the earliest Chinese folk songs around 551-479 BC and in medical books (Erhardt, 1992). Through trade daylilies might have reached Europe where they became further popular and improved through hybridisation and selective breeding (Stout, 1934). The first hybrid of daylily obtained by crossing *Hemerocallis* species was developed around 1890 in England. Immigrants from Europe brought first daylilies, the Lemon daylily, *H. flava* and the Tawny daylily, *H. fulva*, to America where they have been bred extensively to develop modern daylily cultivars.

Although there is no documented evidence, it is believed that daylilies were introduced to New Zealand during the 1800's by Chinese immigrants who arrived in Otago and West Coast of the South Island as gold diggers (Gear, 1998). The modern daylilies grown in New Zealand are mainly raised in America and to a lesser extent in Australia. As a garden plant daylilies has a great potential and its popularity keeps spreading to other countries including tropics. Although most of the daylily breeders are found in the United States, there are breeder groups in Canada, Great Britain, Australia, New Zealand, Germany, Japan, Korea and China.

1.4.2 Physical Characteristics

Daylilies are herbaceous perennials with an underground stem, which is usually called the 'crown'. The leaves are linear, strongly ribbed and arranged in two ranks that are closely compacted and equitant at the base and that spread gracefully above to form a symmetrical 'fan'. The disposition, height and colour of the leaves vary among species and garden clones. The flower stalks or scapes arise from the crown. They can be branched or unbranched with leaf-like bracts at the nodes. They can be erect, arched or bowed towards the ground. The height and branching pattern of scape can vary among species. Occasionally small axillary shoots called proliferations arise from the flower scape, which can be used for vegetative propagation. Flowers are large and colourful with a perianth of six petals in two whorls (botanically all six are tepals), six stamens and a pistil. The perianth parts are united at the base to form a well-defined tube. The colour of the original daylily flower varied among species from yellow through orange and reddish brown to near red. Each flower normally blooms for one day and there will be a succession of flowers throughout the flowering season. The fruit is a dry dehiscent capsule of three chambers with numerous black, rounded or somewhat angled seeds. The roots are numerous and divided into many branches. Depending on the species, the main roots could be slender and cylindrical, slightly or noticeably swollen.

1.4.3 Classification

The genus *Hemerocallis* to which daylilies belong are grouped under the family Liliaceae by Linnaeus. However, there are clear differences between other lilies in the lily family and daylilies. Unlike other members of the lily family, daylilies neither form bulbs nor form tubers or rhizomes. The roots of daylilies are tapering and may form swellings that act as storage organs. They are different from lilies in the shape of the seeds and the position of the nectaries. Lily seeds are flat and brown while daylily seeds are black and round or ovoid. In lilies nectaries are placed in the base of the perigonial leaves whereas in daylilies they are situated in the walls of the ovaries. Therefore, with the recent reorganisation of the order Liliaceae, most botanists believe that daylilies should be placed in the family Hemerocallidaceae. However, recent molecular DNA studies show that daylilies are most closely related to Phormium, which would place them in the family Phormiaceae, in the order Asparagles (Grenfell, 1998).

Stout (1934) divided the genus *Hemerocallis* into two groups, those with branched scapes (Euhemera) and those without branches (Dihemera). Erhardt (1992) divided the genus into five groups based on root, scape and flower characteristics. Those are Fulva, Citrina, Middendorffii, Nana and Multiflora.

1.4.4 Modern Daylilies

Modern daylilies have been developed by breeding generations of plants. As a result, there is a vast collection of daylily cultivars, well over 32,000 (Anthony, 1996). These daylilies have been improved in performance and effectiveness as a garden plant. There have been changes in flower colour, size, shape, colour patterning on the petal, number of petals, colour of the throat, dusting and dotting on the petals to get a sparkling effect in the sun, nature of the edge of the petal, nature of the midrib of the

petals, side and front view of the flower, shape and texture of petals, colour of the anthers, flowering time, flowering season, length and habit of scape, growth habit of plant, length, width and colour of leaf and winter hardiness. These changes to almost every organt of the daylily plant has brought about a specialised terminology to describe each part (Erhardt, 1992; Grenfell, 1998). The choice of colours available at present could vary from near white to yellow, orange, copper, peach or melon, brown, pink, red, lavender, purple and all shades of these colours and intermediate tones. There are flowers of almost blue and almost black. Pure white and pure blue has not yet been achieved, and seems to be the objective of most breeders at present.

1.4.5 Uses of Daylilies

As a garden plant daylilies rank among one of the most valuable herbaceous perennials. Daylilies thrive in any soil and in any part of an ordinary garden. With their strong fleshy roots they can withstand dry seasons to a certain extent. Due to their compact, sturdy habit of growth, garden weeds cannot crowd out daylilies. Apart from proper planting and transplanting together with occasional tidying up daylilies do not require much attention. They are also quite hardy and are resistant to diseases and insect pests are normally infect other garden plants (Stout, 1934; Erhardt, 1992). Different species and clones show a wide diversity in habit of growth, season of flowering, and in size, shape, colour and fragrance of the flowers which makes daylily suitable to enhance the style, design and size of any garden.

Although the usefulness of daylily as a cut flower is doubtful due to its short life span, it could be used in floral display when appropriate measures are taken. Normally double-petalled daylily flowers last longer than single petalled daylilies making the former more suitable for vases and other floral arrangements. Besides, those with a fragrance will give an added value when used in interior decorations.

Daylily flowers are used extensively as a delicacy among Chinese. In some parts of China daylilies are cultivated as a crop plant to produce flowers for food. In New Zealand commercial products of dried daylily flowers are sold in Chinese food markets under the name '*gin-zhen-chi*' (meaning golden needles). The edible, dried daylily flowers have been shown to contain 2.27% fat, 11.42 % proteins, 3.3% minerals and 8.48% fibre together with high quantities of Vitamin A and B (Hu, 1964).

The medicinal use of daylily is also extensive. In China it is used as an antidote to poisons and as an antidepressant. It is also used to cure breast abscesses, dropsy, anuria, intestinal bleeding, dysuria, lithiasis, gonorrhoea, jaundice and piles. A soup made by boiling daylily roots with pork is believed to promote formation of blood cells, provide strength, and relieve fever and toothache (Hu, 1964).

To this day the dried foliage, which is quite tough, is plaited into cords and then used to make footwear in some parts of Asia.

1.4.6 Propagation of Daylily

Traditionally daylilies have been propagated vegetatively by dividing compound rhizomes and multiples stems (Stout, 1934). After dividing, roots and leaves are trimmed to about half their length before they are replanted. As the popularity of daylilies increased, improved methods of vegetative propagation have been introduced by various authors (Traub, 1937; Norman, 1964; Apps and Heuser, 1975; Erhardt, 1992).

Daylilies can also be propagated by planting proliferations that develop on flower scapes. Normally, proliferations are planted with a section of the parent scape. Many

types of daylilies never produce proliferations, but various hybrids and polyploids produce them abundantly, in which case they may be used for rapid propagation.

Multiplication of daylilies using seeds is faster that multiplication using vegetative organs. However very few daylilies reproduce true to type by seeds. Besides, many new hybrids and clones are sterile making it impossible to propagate by seeds. However when available, seeds could be used for propagation of daylilies in large numbers. For example, a single plant of Lemon daylily may yield several hundred seeds (Stout, 1934). Mature plants may be obtained two to three years after germinating such seeds.

1.4.7 Tissue Culture of Daylily for Micropropagation

Although thousands of new daylily hybrids with improved characteristics have been produced using various hybridisation techniques, many of these cultivars have been remained in breeder and collector gardens unknown to the general gardening public as a result of slow multiplication of the plant (Apps and Heuser, 1975). Hence there was the need to develop faster methods to regenerate daylilies of the same clone in large numbers. In order to fulfil this requirement micropropagation of daylilies by tissue culture has been first attempted in 1970's. Mullin (1970) induced callus tissue from tuberous roots of *Hemerocallis* species. However, development of plantlets from callus tissue had not been observed. In 1972, Chen and Holden reported production of daylily (*Hemerocallis fulva* L.) plantlets by tissue culture using petals. They were able to induce adventitious roots from petal explants grown in Murashige and Skoog media (MS) containing 6 mg/L NAA in dark at 25°C. These roots when transferred to MS containing 5 mg/L 2,4-D produced callus, which eventually resulted in shoots in media supplemented with 2,4-D and kinetin. Then the plantlets were transferred into growth regulator deficient MS media for further development. In the cultivar 'Chipper Cherry' callus was induced from petal explants first in MS containing 2,4-D and

kinetin in dark from which plantlets were produced in media with no 2,4-D but with IAA and kinetin under 16 h photoperiod (Apps and Heuser, 1975). In the same cultivar, short lived callus was induced in a similar medium with 2,4-D and kinetin using floral stalks as explants, but no callus was induced from tuberous root explants (Heuser and Apps, 1976). This group also tested liquid culture media to increase quantity of callus derived from petal explants from buds. Several tetraploid daylily cultivars produced callus in high salt MS medium supplemented with 10 mg/L NAA and 0.1 mg/L kinetin in dark from inflorescence slices (Meyer, 1976). These callus tissues resulted in plantlets in similar media with 0.5 mg/L NAA and 0.1 mg/L kinetin. The plantlets were reported to produce true-to-type flowering plants when transferred to soil. Large-scale production of daylily variety 'Azetec Gold' by tissue culture in a three-stage process is described by Strode and Oglesby (1976) using shoot tips as explants. The stages involved were, establishing plants in sterile culture in MS, rapid multiplication in MS with IAA (0.2 mg/L) and 2iP (25 mg/L), and rooting in MS with IAA (10 mg/L) and NAA (2 mg/L). This programme is reported to produce 1000 plantlets each week. Five diploid cultivars of daylily using ovary explants have been tried in MS medium with 2,4-D and BA (Krikorian and Kann, 1979). Relatively high levels of 2,4-D (10 mg/L) with relatively low levels of BA (0.001-0.1 mg/L) and vice versa, i.e. lower levels of 2,4-D (0.001-0.1 mg/L) with higher levels of BA (0.1-1 mg/L) produced callus. However equal amounts of the two growth regulators failed to yield callus. The callus obtained could be easily subcultured every 30 days using large (0.5-1 cm diameter) inocula. Shoots and roots were produced from these calli when transferred to media containing lower amounts of BA and 2,4-D. In this study, the minimum time taken to pot plants in the greenhouse from the initiation of sterile cultures was about 135 days where as a procedure that increased plantlet yield using higher levels of 2,4-D and BA took about 180 days.

By introducing an additional step using liquid culture, it had been possible to increase the yield further while reducing the minimal size of the inoculum needed to get organised growth. Such suspension cultures can be routinely maintained by

subculturing (Krikorian and Kann, 1979; Krikorian and Kann, 1979; Krikorian and Kann, 1980; Krikorian and Kann, 1981). Subculturing was done using inocula of cell aggregates obtained by passing suspensions through a double layer of cheesecloth or sieves of 140 μm-864 μm (Krikorian and Kann, 1981). Cell aggregates grew into large, round clumps when grown in MS medium with no added 2,4-D and, subculturing was carried out in modified White's medium supplemented with 200 mg/L casein hydrolysate. These clumps developed into organised structures in 3 weeks when transferred into Schenk and Hildebrandt medium (Schenk and Hilderbrandt, 1972). Shoots and roots were formed from organised structures after 6 weeks in the same medium in semi-solid form supplemented with 100 mg/L inositol. The plantlets thus obtained could be successfully transplanted into soil. These plants produced flowers 2 years after introducing into soil (Krikorian and Kann, 1980). Both plants and flowers thus produced were morphogenetically similar to those propagated by conventional means (Krikorian and Kann, 1980; Krikorian and Kann, 1981).

Morphogenetically competent cell suspension cultures of daylily initiated using media containing growth regulators can be maintained and multiplied as preglobular stage proembryos (PGSPs) in growth regulator-free, low pH media with low levels of nitrogen, provided they have been in media containing growth regulators for at least 12 weeks (Smith and Krikorian, 1991). PGSPs can be maintained and multipled on liquid or semi-solid growth regulator-free media at pH 4.5. PGSPs in semi-solid media could be maintained up to 1 year without any loss of capability for embryogenesis. When PGSPs are cultured on activated charcoal-impregnated filter papers in media with reduced nitrogen and buffered at pH 5.8, they developed into mature somatic embryos. However the embryos that did not show proper development at the early stages did not germinate. There were about 20% of such embryos, especially when activated charcoal was not used. Sometimes secondary somatic embryos emerged from primary embryos. These secondary embryos germinated into plantlets even if they were first developed on aberrant primary embryos. The quality of the embryos produced according to the above procedure

varied with the dimensions of the units from which they were derived. The larger fractions yielded more multiple somatic embryos and responded at a faster rate than finer ones (Krikorian et al., 1995).

In vitro regeneration of daylilies by protoplast culture has been tried (Fitter and Krikorian, 1981; Krikorian, et al., 1982; 1988; Ling and Sauve, 1995). This should enable somaclonal variation, somatic hybridisation and genetic transformation techniques be utilized to improve cultivars so that sexual incompatibility among cultivars and heterogenous parental material would not restrict the production of new daylily cultivars (Ling and Suave, 1995). Protoplasts derived from cell suspension cultures of daylily appeared to regenerate cell walls easily and more easily undergo subsequent divisions when compared to cells obtained from mature plants or aseptically grown organised plant parts (Krikorian et al., 1988). When cells are obtained from mature parts, they are in a wide range of maturity and differentiation states making it difficult to obtain stable morphogenetically competent cells for manipulation. On the other hand, suspension cultures provide ample materials that are competent for protoplast isolation, cell wall regeneration and formation of plants. With the cv. Autumn Blaze, hundreds of plants have been generated via protoplast culture (Fitter and Krikorian, 1981), which were phenotypically and physiologically similar to parent plants from which the explants were obtained (Krikorian et al., 1982). Protoplasts derived from leaf mesophyll cells of *Hemerocallis* × red magic were successful in producing plantlets when compared to those derived from unorganised suspension cultures (Ling and Sauve, 1995).

1.5 AIMS AND OBJECTIVES

Of the three types of flower senescence described by Woltering and van Doorn (1988), majority of the studies on flower senescence is carried out on ethylene-sensitive ones. There is a considerable understanding about the genetic basis and processes involved in senescence of those flowers. However, only a few families such as Campanulaceae, Caryophyllaceae and Malvaceae (Woltering and van Doorn, 1988) show ethylene-sensitive flower senescence. Flower senescence in families such as Compositae, Iridacae and Liliaceae is insensitive to ethylene (Woltering and van Doorn, 1988). In addition, senescence of most of the cut flowers, for example, iris, tulip, narcissus are ethylene insensitive (Reid, 1989). Because of these reasons, ethylene-insensitive flower senescence has attracted the interest of scientific world recently.

Daylily has become popular for the study of ethylene-insensitive flower senescence due to its short flower life. The entire process from flower opening to wilting of petals is completed within a single day. At present, there is a general agreement among several studies on daylily petal senescence showing that protein degradation (Lay-Yee et al., 1992; Stephenson and Rubinstein, 1998), increase in proteinase activity (Stephenson and Rubinstein, 1998) and up-regulated expression of genes coding for proteinases (Valpuesta et al., 1995) are all correlative events occurring after flower opening and have become particularly prominent at the late stages of flower senescence. In daylily, petal senescence is associated with thioproteases or endopeptidases (Guerrero et al., 1998; Stephenson and Rubinstein, 1998). This observation is supported by both the nucleotide sequence data and the data from enzyme activity against gelatin or azocasein as substrate

Besides endopeptidases, carboxypeptidases and aminopeptidases could also contribute to protein breakdown during flower senescence. Measurements of these exopeptidase activities have not been reported in daylily petals. It has been generally

assumed that aminopeptidases occur in virtually all plant tissues including different floral parts (Walling ang Gu, 1996). There is one published study, using synthetic peptide substrates that measured aminopeptidase and carboxypeptidase activities in the tulip petal extracts (Sopanen and Carfantan, 1976).

The physiological role of carboxypeptidase activity in petal extracts has received scant discussion. Aminopeptidases are likely to participate in the general events of plant cell protein turnover (Walling et al., 1996). However, it is also possible that some aminopeptidases could have more specific roles to play in a plant's life cycle. The occurrence of LAP activity during flower development might be related to the hypothesis that it could be a plant defence protein (Walling et al., 1996). It is also possible that the development of LAP activity is closely associated with senescence of various organs including petals. These possibilities will be evaluated here using daylily petal extracts of cv. Stella D'oro.

The presently available classification based on pH optima recognises two types of aminopeptidases in plants, i.e. neutral and alkaline types. Characterisation of LAP from daylily cv. Stella D'oro will be attempted in this study, with the hope of shedding some more light on the properties of the enzyme activity in daylily petals (cv. Stella D'oro).

Most of the changes that are taking place during senescence of plants are secondary or peripheral (Lesham et al., 1986). Senescence is a developmentally regulated programmed process, which is hypothesised to be governed by the genetic basis of the plant (Nooden et al., 1997). There are external and internal factors that could affect the natural senescence process. These factors may have an influence upon the central mechanism of senescence. The relevance of LAP enzyme to daylily flower senescence is studied using numerous compounds, besides those involved with ethylene production/action aspects that might affect the longevity of petals. Although it is very interesting, it is not known whether the senescence of daylily leaves is

ethylene-insensitive or not. This will be investigated here and extended to include a comparison of the effects of numerous compounds on both leaf and flower senescence of daylily cv. Stella D'oro. The regulation of LAP in daylily flowers has been explored preliminarily at the RNA and DNA level.

Daylily is a very popular landscape plant all over the world. It has been improved tremendously through selective breeding to produce many cultivars and is commercially propagated by tissue culture (Chu and Kurtz, 1990). Although a considerable number of literature is available on micropropagation of daylilies, there are certain aspects of microprpagation of daylily which have not been looked into or not reported. Although some of the published journal articles on the subject claim to have produced large numbers of plantlets, surprisingly quantitative results are not available on micropropagation of daylilies.

The main objective of developing a micropropagation technique for tissue culture of daylily is to get a large number of plants of daylily rapidly so that enough plant material, especially flowers from clonal plants will be available for studies on senescence. While trying to develop the micropropagation technique, I have tried to get information on the aspects that have been neglected on daylily micropropagation in the literature available. It is generally understood that the position of the explant in the tissue used for culturing has an effect on the subsequent success of the microprpagation technique. This aspect of daylily has not been tested in the literature available. Therefore several experiments were designed to study the suitability of different floral parts as explants for micropropagation, the effect of the position of the explant in the appropriate tissue before culturing on the subsequent *in vitro* responses, and to document the results obtained in a quantitative manner.

In selecting growth regulators for micropropagation, combinations of NAA and BA or NAA and 2iP in different ratios had been used. Use of these growth regulator combinations in micropropagation of daylily is novel to this thesis.

The general practice in tissue culture is to add a carbon source, usually sucrose, to the growth media to support tissue or plant growth. However, this may not be always essential. The effect of sucrose on rooting and subsequent growth of daylily was investigated.

In summary, the main objective of this thesis is to study the role of proteolytic enzymes, particularly that of LAP in ethylene-insensitive flower senescence using daylily as the model system.

Micropropagation of daylily were attempted using four daylily cultivars, namely Stella D'oro, Siloam Ribbon Candy, Chorus Line and Dallas Star. Of these four cultivars, cv. Stella D'oro was the first to be tested for micropropagation and therefore the first to produce plantlets. Hence, the plantlets obtained from this cultivar were the only ones transferred to soil to grow on for flower production and provided flowers in large numbers. Due to this reason, almost all the experiments related to daylily flower senescence had been carried out using this cultivar.

CHAPTER 2

MATERIALS AND METHODS

2.1 PLANT MATERIAL

2.1.1 Maintenance of Daylily Plants in a Glasshouse or Growth Room

Initially, a few plants of daylily (*Hemerocallis* sp.) cvs. Stella D'oro, Siloam Ribbon Candy, Chorus Line, Dallas Star, Black Eyed Stella and So Sweet were obtained from the Taunton Gardens, Christchurch, New Zealand. They were grown in potting mix (containing 60% bark, 20% peat, 10% sand and 10% sterilized soil) with 8-9 month slow release fertiliser granules in the glasshouse (25-30/15-20°C day/night during summer and 15-20/10-15°C day/night during winter) under 16 h photoperiod (sodium light, 400w) at the University of Canterbury. Each plant was divided into 3-5 parts and repotted in new potting mix in the winter. Some of the tissue cultured plants obtained in the course of this study were grown in a growth room at 22°C under 16 h photoperiod (sodium light, 60w) for flower production during winter months. Plate 2.1 shows the fully open flowers of cv. Stella D'oro, Siloam Ribbon Candy, Chorus Line, Dallas Star, So Sweet and Black Eyed Stella on plants originally obtained from the Taunton gardens.

2.2 MICROPROPAGATION OF DAYLILY

2.2.1 Types of Explants

Outer petal, inner petal and ovary explants obtained from flower buds of cv. Stella D'oro, Siloam Ribbon Candy, Chorus Line and Dallas Star were tested for the

initiation of shoot primordia *in vitro*. Explants from two different bud sizes described as medium size and small size (defined in Table 2.1) were tested from each cultivar.

2.2.2 Culture Initaition

Flower buds of the appropriate size were harvested, surface sterilised for 15 min in a diluted commercial bleach solution (Dynawhite, New Zealand) containing 1% sodium hypochloride and rinsed 5 times in sterile distilled water. The pedicels were removed from the buds and dissected carefully to separate floral parts. Each outer and inner petal, of the medium-sized flower buds, were cut horizontally into three equal segments, while those of small-sized buds were cut into two equal segments. The flower tube was divided into 2 segments, lengthwise, and included under the category of outer petals. The ovaries were also cut into two equal parts lengthwise. Three replicate buds from each size were tested in each combination of growth regulators. Hence, the number of explants from inner petals, outer petals and ovary of the medium-sized buds were 27, 33 and 6 respectively. Small-sized buds had 18, 24 and 6 inner petals, outer petals and ovary explants respectively.

The cv. Stella Dóro had been used to test whether there is an effect of the position in the petal from which the explant was obtained in producing shoot primordia-like structures (tiny, green buds that mature into shoots later on). The inner petal explants from medium-sized buds were categorised into 3 groups, namely explants from the base of the petal, explants from the middle of the petal and explants from the tip of the petal (9 explants for each category). Outer petal explants were categorised into similar groups (9 explants from each category) but with an additional group where the explants came from the tubes (6 explants) of the bud. The petal explants from small-sized buds were grouped as those from the base and the tip of the petal. The two explants from the tubes were included as a separate group among the outer petal explants. There were 6 explants for each group from small-sized buds. The explants thus prepared were placed in 50 mL of the medium described in 2.2.3.2 in 50 mL or

200 mL plastic tissue culture containers (Biolab Scientific, New Zealand) each with four explants. All the manipulations were carried out under aseptic conditions inside a laminar flow cabinet (Airpure Email Westinghouse Pty Ltd, Australia). Three buds from each bud size were tested in each combination of growth regulators. All the containers were kept in a growth room maintained at 22°C with a supply of constant cool white light at an intensity between 120-145 $\mu E/m^2s^{-1}$. Observations were first taken at 2 weeks after inoculation and at 4-6 week intervals thereafter, for the production of shoot primordia-like structures.

2.2.3 Media

2.2.3.1 Media Preparation

Half strength Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) was prepared from stock solutions of major salts, minor salts, iron and organic supplements (Appendix 1, Tables A-D). Required volumes of plant growth regulators were added from stock solutions of 100 mg/mL. The carbon source, sucrose (Chelsea, NZ Sugar Co Ltd, New Zealand) was dissolved and the pH adjusted to 5.6-5.8 using 0.1 M HCl (BDH, UK) or NaOH (BDH). The total volume was adjusted prior to adding agar (Germantown NZ Co., New Zealand) and being heated in a microwave oven with occasional stirring. Upon boiling, media were dispensed as 20 mL or 50 mL aliquots into 50 mL or 200 mL plastic tissue culture containers respectively, and autoclaved for 15 min at 147 kPa pressure.

Black Eyed Stella

Chorus Line

Stella D'oro

Dallas Star

Siloam Ribbon Candy

So Sweet

Plate 2.1
The flowers of daylily cultivars grown in the glasshouse of the University of Canterbury for this study.

Table 2.1: Description of the two sizes of floral buds used to obtain explants for micropropagation of daylily cultivars. Errors represend standard deviation for three flowers.

Cultivar	Length of flower bud (cm)	
	Medium Size	Small Size
Stella Dóro	2.0 ± 0.3	3.5 ± 0.3
Dallas Star	3.0 ± 0.3	5.0 ± 0.3
Chorus Line	2.0 ± 0.3	3.5 ± 0.3
Siloam Ribbon Candy	2.0 ± 0.3	4.0 ± 0.3

2.2.3.2 Media Tested for Shoot Initiation

Half strength MS with 3% sucrose and 0.8% agar with α-naphthalene acetic acid (NAA) (Sigma, USA) and isopentyleaminopurine (2iP) (Sigma) or benzylaminopurine (BA) (Sigma) were used for shoot initiation. Three concentrations of NAA were tested with three concentrations of either 2iP or BA as follows: 5 mg/L NAA and 1 mg/L 2iP, 3 mg/L NAA and 3 mg/L 2iP, 1 mg/L NAA and 5 mg/L 2iP, 5 mg/L NAA and 1 mg/L BA, 3 mg/L NAA and 3 mg/L BA, 1 mg/L NAA and 5 mg/L BA.

2.2.3.3 Media for Root Initiation and Maintenance of Shoots and/or Plantlets

Explants that produced shoot primordia-like structures on shoot initiation media were transferred to 20 mL of half strength MS with 3% sucrose and 0.8% agar in 50 mL tissue culture containers for root initiation.

Plantlets were maintained in 20 mL of the same medium either in 50 mL or 200 mL tissue culture containers. Subculturing was carried out every 3 months. Clusters of plantlets (Plate 2.2A) were separated into single plantlets or small clusters of about 5 plantlets during subculture. All the containers were incubated under the conditions described in section 2.2.2.

2.2.4 Establishment of Tissue Cultured Plantlets in Soil

Twenty plantlets of cv. Stella Dóro, which had been subcultured for 4-8 times and were about 7-8 cm tall (Plate 2.2B), were selected randomly for planting out in soil. The selected plantlets were transferred into new subculturing medium and incubated for two weeks under the conditions described in section 2.2.2. Then the plantlets were

removed from tissue culture containers, and their roots were washed in water to remove any agar before they were planted in 20 g of sterile potting mix described in 2.1.1 with 6-8 months old slow release fertiliser granules in 6.5 × 6.5 × 9.0 cm plastic pots. Pots were watered, completely covered with transparent plastic bags to retain humidity at 100% and kept in a growth room with 16 h photoperiod and at 22°C. After 15 days, plastic bags were opened slightly so that plantlets were exposed to the outside air. In twenty days, the plastic bags were removed completely. Forty three days later, plantlets established in sterile potting mix were transferred to potting mix in black polythene bags (PB 18, Epic, New Zealand) and kept in the glasshouse thereafter. The planting out experiment had been carried out with a second set of 20 plantlets of the same cultivar to confirm results.

2.2.5 Effect of Sucrose Concentration on Rooting

Plantlets of cv. Stella Dóro that had been subcultured for 4-8 times and about 7-8 cm tall (as shown in Plate 2.2B) were used for this experiment. Before the experiment, plantlets were transferred into new subculturing medium and incubated for two weeks under conditions described in section 2.2.2. Then the plantlets were removed from the tissue culture containers, the roots were cut off completely and the leaves were trimmed to make the plantlets 6 cm tall. After this they were transferred to 50 mL of 1/2 strength MS with 0.8% agar and different sucrose concentrations. The sucrose concentrations tested were 0, 3 and 6%. Six replicate plantlets (1 plantlet per container) were used in each sucrose concentration. The number of roots and their morphology were recorded every week for 6 weeks. At the end of 6 weeks, the plantlets were transferred to soil as described in section 2.2.4. The experiment was duplicated.

A

B

Plate 2.2
Tissue culture of daylily cv. Stella D'oro for micropropagation. A: Cluster of plantlets in rooting medium. B: Individual plantlets in subculture and ready to be transferred to soil.

2.3 PROTEINS ASSOCIATED WITH DEVELOPMENT AND SENESCENCE OF DAYLILY PETALS

2.3.1 Harvesting of Flowers, Leaves and Roots

Flowers or flower buds of the cv. Stella D'oro at the appropriate stage were harvested from the plants growing in the glasshouse at 9 a.m. each morning for most of the experiments unless specified otherwise. Buds (slightly open at the tip with the stigma still inside the bud) were either harvested or labelled the day before full bloom to obtain flowers developed from buds at the same stage of development. However, buds collected 2 days before opening were not labelled. Petals from the buds and flowers were separated, weighed, frozen in liquid nitrogen and stored at -80°C until further use.

Green leaves and roots of cv. Stella D'oro were collected from tissue cultured plantlets growing in half strength MS as described in section 2.2.2. Roots and leaves were washed to remove adhered agar, blotted dry, weighed, frozen in liquid nitrogen and stored at -80°C for later use. For the experiments testing longevity of leaf discs in different chemicals, leaves were collected from glasshouse grown plants of cv. Stella D'oro.

2.3.2 Extraction of Proteins from Plant Material

In the case of flowers, all the petals and flower tubes were combined for protein extraction except when studying enzyme levels separately in inner petals, outer petals and tubes. The tissues were grounded into a fine powder using liquid nitrogen in a cold mortar and a pestle. Powdered tissues were transferred to 50 mL centrifuge tubes

before cold phosphate buffer were added (0.01 M) at pH 7 (Appendix 2, Table A), mixed and left for 30 min in ice for protein extraction. Two and half mL of extraction buffer were added per 1 g of tissue. After extraction, the mixture was filtered through double layers of synthetic cloth and centrifuged for 30 min at 11,000 rpm and 1-4°C (Centrifuge 5403, Eppendorf, Germany). The supernatant was transferred to a measuring cylinder and brought to a known volume using cold extraction buffer, mixed and stored in Eppendorf tubes at -20°C.

For leaves and roots, protein extractions were carried out in the same way as for the petals.

2.3.3 Determination of Total Protein Concentration

Total protein concentrations in the extracts were determined according to Bradford (1976). One hundred μL of the protein extract were mixed with 1 mL of Coomassie Brilliant Blue G (Sigma) dye reagent, and incubated for 5 min at room temperature before the absorbance was measured at 595 nm (Pharmacia Biotech, Novaspec II, Sweden). The extraction buffer was used as the diluent in cases where dilution of the protein extract was needed. Distilled water had been used as the blank. A standard curve had been prepared with 0-100 mg/L bovine serum albumin (BDH).

2.3.4 Effect of Storage at Low Temperature on the Stability of Proteolytic Enzymes

2.3.4.1 LAP

Enzyme extracts from petals of fully open flowers of the cv. Stella D'oro, collected on the day of opening, were stored in Eppendorf tubes as 1 mL aliquots at -20°C and

used as required to study the effect of storage time on LAP activity. To study the effect of repeated freezing on LAP activity, enzymes extracted from the same stage were subjected to repeated freezing at -20°C and thawing for 4 times on the day of extraction and were analysed for LAP activity after thawing each time. Activity of the fresh extract was considered as 100%.

Effect of cold storage on isozymes was studied by IEF using fresh extracts and once frozen (at -20°C) extracts from buds collected on the day before opening, fully open flowers collected on the day of opening and senesced flowers collected on the day after opening of cv. Stella D'oro.

To study whether there was an effect of storing petals at -80°C on enzyme activity, fresh enzymes extracted from petals of fresh flowers and flowers after being stored at -80°C were also subjected to IEF as described in 2.3.10.3.

2.3.4.2 Endopeptidase and Carboxypeptidase

Fresh and once frozen (at -20°C) extracts from full bloom flowers collected on the day of opening and senesced flowers collected on the day after opening were used for the determination of effect of low-temperature storage on carboxypeptidase and endopeptidase activity respectively (the appropriate enzyme assays described in 2.3.8.1 and 2.3.9.1).

2.3.5 Determination of the Presence of Endogenous Proteolytic Enzyme Inhibitors and Promoters in Petal Extracts

Enzyme extracts were prepared as described in section 2.2.2 using petals from buds and flowers of cv. Stella D'oro collected at different stages of development. Inhibitor

or promoter activity was determined by preincubating at 37°C a volume of an extract with the same of that from another stage of development, both separately and together (Halaba and Rudnicki, 1983). At the end of 30 min of incubation the remaining LAP activity was measured (as described in 2.3.7.1), endopeptidase and carboxypeptidase enzymes (as described in 2.3.8.1 and 2.3.9.1). A theoretical value of enzyme activity for the combined extracts was calculated by adding the activities of the extracts incubated alone under the same conditions. The difference between the actual value and the theoretical value of the mixed extracts was divided by the actual value and expressed as a percentage to get a measure of inhibition or promotion.

2.3.6 Presence of Low pH Extractable Proteolytic Enzymes in Petals, Leaves and Roots

Petals from buds collected on the day before opening, fully open flowers collected on the day of opening, senesced flowers collected on the day after opening, leaves and roots of cv. Stella D'oro were used in this experiment. Low pH extractable proteins were extracted using citrate phosphate buffer (1 mM) at pH 2.8 (Appendix 2, Table B). Three samples from each stage were ground to a fine powder and divided into 2 equal portions based on weight. One portion was extracted with pH 2.8 buffer while the other portion was extracted with 0.01M pH 7 phosphate buffer as described in section 2.3.2. All the extracts were dialysed against 0.01M pH 7 phosphate buffer overnight. Then the extracts were centrifuged and assayed for the appropriate enzyme activities. Total proteins in the extracts were determined using the Bradford method described in section 2.3.3.

2.3.7 LAP

2.3.7.1 Determination of LAP Activity

LAP activity was determined according to Leung and Bewley (1983) using fresh extracts. The substrate solution was prepared by dissolving 10 mg of L-leucine ρ-nitroanilide (Sigma) in 0.5 mL of dimethyl sulfoxide (Sigma) and adjusting the volume to 10 mL with phosphate buffer (0.01 M) at pH 7. The reaction mixture contained 0.3 mL of substrate solution, 0.32 mL of phosphate buffer at pH 7 (0.01 M) and 10 µL of enzyme extract in Eppendorf tubes. The control tubes contained 0.32 mL of sodium phosphate buffer and 10 µL of enzyme extract. All the tubes were kept on ice. The pH of the mixture was 7. The tubes were vortexed and incubated at 37°C for 30 min in a water bath. After the incubation time, the tubes were placed on ice and 0.3 mL of substrate solution was added into control tubes. Then the tubes were boiled for 5 min to stop the reaction. After this, 0.67 mL of distilled water was added into the tubes and absorbance was measured at 410 nm. One unit of enzyme activity is expressed as a change in one unit of absorbance per h per flower, per g fresh weight of petals or per mg soluble proteins in the extract.

2.3.7.2 LAP Activity in Daylily Petals during Development of Flowers

The time course study on the LAP activity on petals of the cv. Sella D'oro had been carried out using buds collected at 9 a.m. and 9 p.m. on the day before opening, fully open flowers collected at 9 a.m., 3 p.m. and 9 p.m. on the day of opening and fully senesced flowers collected at 9 a.m. the following day. Fully open flowers collected at 9 a.m. on the day of opening were considered to have been collected at 0 h.

For comparison among different cultivars of daylily, petals from three distinct developmental stages namely, bud stage (collected 2 days before opening) (described in Table 2.2), full bloom flower stage (collected on the day of opening) and senesced

flower stage (collected on the day after opening) were used. The cultivars studied were Stella D'oro, Siloam Ribbon Candy, Chorus Line, Dallas Star, Black Eyed Stella and So Sweet.

2.3.7.3 Effect of pH on LAP Activity in Petals and Leaves

The enzyme activity was determined as described in 2.3.7.1 at pH values 6, 7, 8, 9 and 10 using crude enzyme extracts from fully open flowers collected on the day of opening, and leaves of cv. Stella D'oro. To obtain the desired pH, the reaction buffer (0.01 M phosphate buffer at pH 7) was replaced by either 1 mM citrate phosphate buffer or 2 mM Tris HCl buffer. Mixing the substrate with citrate phosphate buffer at pH 5.2 and 7.2 (Appendix 2, Table B) resulted in final pHs of 6.1 and 7.0, respectively while mixing it with Tris HCl buffer at pHs 8.7, 9.6, and 9.8 (Appendix 2, Table C1) resulted in final pHs of 7.9, 8.7 and 9.8, respectively.

2.3.7.4 Effect of Temperature on LAP Activity

Effect of temperature on LAP activity in petals of cv. Stella D'oro was determined as described in section 2.3.7.1 at 37, 50, 60 and 70°C using crude petal extracts from fully open flower. In a separate experiment, the enzyme extracts were preincubated at the testing temperature for 5 or 10 min with or without 0.5 mM $MnSO_4.4H_2O$ (manganese sulphate) (BDH) before assaying for the enzyme activity at 37°C.

Table 2.2: Description of bud stage of different cultivars of daylily used for the determination of LAP activity.

Cultivar	Description of bud
Stella Dóro	Greenish yellow, tightly closed buds of 5.0 ± 0.2 cm long
Dallas Star	Yellowish green, tightly closed buds of 7.5 ± 0.2 cm long
Chorus Line	Yellowish green, tightly closed buds of 4.0 ± 0.2 cm long
Siloam Ribbon Candy	Reddish green, tightly closed buds of 4.0 ± 0.2 cm long
So Sweet	Yellow, tightly closed buds of 6.0 ± 0.2 cm long
Black Eyed Stella	Greenish yellow, tightly closed buds of 6.0 ± 0.2 cm long

2.3.7.5 Effect of Different Classes of Proteolytic Enzyme Inhibitors and Promoters on LAP Activity

Crude petal extracts from full bloom flowers of cv. Stella D'oro were preincubated with the testing solution at 37° for 30 min in the presence of 0.01 M phosphate buffer at pH 7 and were analysed for LAP activity. The chemicals tested were leupeptin hydrochloride (Sigma), NEM (N-ethylmaleimide) (Sigma), iodoacetamide (Sigma), PMSF (Phenyl methyl sufonyl floride) (Sigma), EDTA (Ethylenediamine-tetra-acetic acid) (BDH), bestatin (Sigma), $MnSO_4.4H_2O$, $MgSO_4.7H_2O$ (magnesium sulphate) (BDH) and $ZnSO_4.7H_2O$ (zinc sulphate) (BDH).

Stock solutions of leupeptin (10 mM), iodoacetamide (10 mM), EDTA (0.1 M), bestatin (2 mM), $MnSO_4.4H_2O$ (1 M), $MgSO_4.7H_2O$ (1 M) and $ZnSO_4.7H_2O$ (1 M) were prepared in distilled water, if required by heating. The stock solution of NEM (1 M) was prepared by dissolving the powder in a minimum amount of ethanol and diluting with water while that of PMSF (0.5 M) was prepared in dimethyl sulfoxide.

2.3.7.6 Effect of Damaging Floral Parts on LAP Activity in Petals

Effect of damaging floral parts was tested by wounding slightly open buds of cv. Stella D'oro at 6 p.m. on the day before opening and collecting the petals of fully open flowers at 9 a.m. the next day for LAP analysis. Damaging flowers were done by cutting 0.2 cm of the stigmatic tip, removing all the anthers, or making a 0.5 cm long cut on one of the inner petals. Undamaged flowers were used as controls.

2.3.7.7 Effect of Different Chemicals on Bud Opening, Longevity and LAP Activity in Buds, Flowers, Petals and Leaves of cv. Stella D'oro

Buds and fully open flowers harvested at 9 a.m. in the morning were immediately dipped in distilled water and transferred to the laboratory. The cut end of the pedicel was trimmed and buds or flowers were dipped in 1.5 mL of distilled water (control) or the testing solution in Eppendorf tubes. The tubes were covered with parafilm and incubated at 25°C and continuous illumination (120-145 $\mu E/m^2S^{-1}$). Observations were taken on the following day. If needed, flower petals were harvested and analysed for LAP activity.

Experiments with detached petals were carried out in the same way using plastic vials covered with parafilm. A slit was made in the parafilm cover to insert petals.

Experiments with leaves were carried out using 20 cm long leaves from glasshouse grown plants. Six cm from the tip, 2 leaf circles were punched from either side of the mid rib using 0.5 cm (diameter) cork borer. Cutting was carried out under water. Two leaf discs were randomly selected from a pool of leaf discs to incubate at 25°C in the dark in 1 mL of distilled water or testing solution in capped glass vials. Observations were taken every day for 14 days. When required, leaf discs were harvested to analyse for LAP activity. Harvesting was carried out when 50% of the leaf discs in the control or in the testing solution turned yellow. Harvested discs were rinsed in distilled water, blotted dry, weighed and frozen in liquid nitrogen before storing at -80°C for later use.

The chemicals tested were cycloheximide (Sigma), silver thiosulfate (STS) (Sigma), ascorbic acid (Sigma), casein hydrolysate (Sigma), ethrel, glyphosate (Monsanto, New Zealand), 8-hydroxyquinoline citrate (Sigma), sucrose, fructose (BDH), glucose (BDH), IAA (BDH), gibberellic acid (GA_3) (Sigma), abscisic acid (ABA) (Sigma), BA, kinetin (Sigma), $MnSO_4.4H_2O$, $ZnSO_4.7H_2O$ and $MgSO_4.7H_2O$.

Silver thiosulfate stock solution (20 mM or 5 mg/mL) was prepared according to Reid et al. (1980) by mixing stock solutions of 0.1 M silver nitrate ($AgNO_3$) (BDH) (stored in a dark bottle) and sodium thiosulphate ($Na_2S_2O_3.5H_2O$) (BDH) in 1:4 proportion immediately before use. Ethrel was prepared by diluting a stock solution of 13 M (at pH 1.5) using phosphate buffer at pH 7. If required, pH of the ethrel solutions was adjusted with 0.1 M NaOH to 7 to release ethylene. Stock solutions of growth regulators were made by dissolving the powder in a minimum volume 0.1 M NaOH or 0.1 M HCl and adjusting the volume using distilled water. All other chemicals were prepared in water.

2.3.8 Endopeptidase

2.3.8.1 Determination of Endopeptidase Activity

Enzyme activity was determined as described by Sarath et al., (1996) with slight modification. The substrate solution was prepared by dissolving 10 mg of azoalbumin (Sigma) in 10 mL of phosphate buffer at pH 7 (0.01 M). The reaction mixture contained 100 µL of substrate solution, 300 µL of citrate phosphate buffer (1 mM) at pH 5.2 with 2 mM cysteine and 200 µL of enzyme extracted according to section 2.3.2 in Eppendorf tubes. The control tubes contained 300 µL of citrate phosphate buffer (1 mM) at pH 5.2 with 2 mM cysteine and 200 µL of enzyme extract. The pH of the mixture was 6. The reaction and control tubes were kept on ice. All the tubes were vortexed and incubated at 37°C for 5-7.5 hr in a water bath. Then the tubes were transferred to ice, 100 µL of substrate solution were added into control tubes, and 1 mL of 5% (w/v) trichloroacetic acid was added into all tubes. After 10 min in ice to precipitate proteins, all the tubes were centrifuged at 11,000 rpm for 10 min at room temperature before the absorbance was measured at 420 nm. One unit of enzyme activity is expressed as change in one unit of absorbance per h per flower, per g fresh weight of petals or per mg soluble proteins in the extract.

2.3.8.2 Endopeptidase Activity in Daylily Petals during Development of Flowers

The time course study on the activity of endopeptidase enzymes on petals of cv. Stella D'oro was carried out using buds collected at 9 a.m. on the day before opening, fully open flowers collected at 9 a.m., 3 p.m. and 9 p.m. on the day of opening, partially and fully senesced flowers collected at 3 a.m., 9 a.m., 3 p.m. on the day after full bloom and at 9 a.m. on the following day. Fully open flowers collected at 9 a.m. on the day of opening were considered as flowers collected at 0 hr.

2.3.8.3 Effect of pH on Endopeptidase Activity in Petals

Petals from senesced flowers of cv. Stella D'oro collected at 9 a.m. on the day after full bloom were used in this study. The enzyme activity was determined as described in 2.3.8.1 at pH values 3.5, 4, 5, 6 and 7. To obtain a final pH of 3.5, 4, 5, 6 and 7, 100 µL of substrate solution and 200 µL of enzyme extract were mixed with 300 µL of citrate phosphate buffer (1 mM) at pH 2.6, 3.2, 4.0, 5.2 and 7.0 respectively (Appendix 2, Table B).

2.3.9 Carboxypeptidase

2.3.9.1 Determination of Carboxypeptidase Activity

The enzyme activity was determined according to Chrispeels and Boulter (1975). Enzymes were extracted from petals using phosphate buffer (0.01 M) at pH 7 as described in section 2.3.2. One mL of each enzyme extract was dialysed overnight against citrate phosphate buffer (1 mM) at pH 5 for 12 h and centrifuged at 15,000 rpm for 10 min at 1-4°C. The supernatant was used for the determination of enzyme activity. The substrate solution was prepared by dissolving 10 mg of N-

carbobenzoxy-L-phenylalanine-L-alanine (Sigma) in 1 mL of dimethyl sulfoxide before bringing the volume to 10 mL with citrate phosphate buffer at pH 5. The reaction and control tubes contained 100 µL of substrate solution, 100 µL of citrate phosphate buffer at pH 5 and 10 µL of enzyme extract in Eppendorf tubes on ice. All the tubes were vortexed and incubated at 37°C for 30 min in a water bath. The control tubes were placed in a boiling water bath for 5 min before incubating at 37°C. At the end of the incubation time, the tubes were transferred to ice, and 100 µL of 5% (w/v) trichloroacetic acid were added, vortexed and incubated for 10 min on ice, before they were centrifuged for 10 min at 11,000 rpm at room temperature. One hundred µL of the supernatant were mixed with 100 µL of ninhydrin solution, vortexed and kept in a boiling water bath for 15 min. The tubes were immediately transferred to ice and 0.7 mL of 50% ethanol was added, vortexed and the absorbance was measured at 570 nm. One unit of enzyme activity is expressed as mg of amino acids released per h per, per g fresh weight of petals or per mg soluble proteins in the extract.

Ninhydrin solution was freshly prepared by dissolving 200 mg of ninhydrin (Sigma) and 75 mg of hydrindantin (Sigma) in 7.5 mL of 2-methoxyethanol (BDH) and was diluted to 10 mL with 1 mM sodium acetate buffer (Appendix 2, table D) at pH 5.4 as described by Sarath, et al. (1989).

To determine the amount of amino acids released by the enzyme reaction, a standard curve was prepared using 1, 5, 10 and 50 mg/L solutions of glycine (Sigma) in citrate phosphate buffer (1 mM) at pH 5. One hundred µL of each of the standard solution was mixed with 100 µL of ninhydrin solution and treated as for the enzyme reaction mixture. Each standard solution was replicated thrice.

2.3.9.2 Carboxypeptidase Activity in Daylily Petals during Development of Flowers

The time course study of the development of carboxypeptidase activity in petals of the cv. Stella D'oro were carried out using buds collected at 9 a.m. on the day before opening, fully open flowers collected at 9 a.m., 3 p.m. and 9 p.m. on the day of opening and fully senesced flowers collected at 9 a.m. on the following day. Fully open flowers collected at 9 a.m. on the day of opening were considered as flowers collected at 0 h.

2.3.9.3 Effect of pH on Carboxypeptidase Activity in Petals

The enzyme activity was determined as described in 2.3.9.1 at pH values 4, 5, 6 and 7 using petal extracts from fully open flowers of cv. Stella D'oro. Before analysis, 1 mL of the enzyme extract was dialysed overnight at 4°C against citrate phosphate buffer (1 mM) with the corresponding pH value. To obtain a final pH of 4, 5, 6 and 7, 100 µL of substrate solution and 10 µL of enzyme extract were mixed with 100 µL of citrate phosphate buffer at pH 3.6, 5.0, 6.2 and 7.0 (Appendix 2, Table B) respectively.

2.3.10 Gel Electrophoresis

2.3.10.1 SDS PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) of Proteins

Proteins were extracted using phosphate buffer (0.01 M) at pH 7 as described in 2.3.2. The required volume of protein extract was mixed with sample buffer (4:1 ratio) and

boiled for 5 min before loading into the wells. Sample buffer consisted of 20% (v/v) glycerol, 10% (w/v) SDS, 5% β-mercaptoethanol and 0.1% (w/v) bromophenol blue (M & B, UK) in 0.125 M Tris HCl (pH 6.8) (Appendix 2, Table C2).

Gels were prepared on an apparatus based on a low-cost and efficient model developed by Studier (1973). Samples were loaded based on mg fresh weight of the tissue. SDS PAGE was performed with 12.5 % (w/v) discontinuous polyacrylamide resolving gels (100 × 140 × 1.5 mm) (Laemelli, 1970) at room temperature. The current was kept at 65 mA through the stacking gel and 25 mA through the resolving gel with unlimited voltage using a Solstat electrophoresis power supply. The resolving gel contained 12.5% polyacrylamide (Sigma), 0.1% SDS, 0.375 M Tris HCl buffer at pH 8.8 (Appendix 2, Table C2), 0.05% (w/v) ammonium persulphate (Bio-rad, UK) and 0.1% N,N,N'N'-tetra methylethylenediamine (TEMED; Bio-rad). A stacking gel (30 × 140 × 1.5 mm) was made up with 4% (w/v) polyacrylamide containing 0.1% (w/v) SDS, 0.125 M Tris HCl buffer (pH 6.8), 0.05% (w/v) ammonium persulphate and 0.1% (v/v) TEMED. Electrophoresis running buffer contained 37.5 mM Trizma® base (Tris) (Sigma), 0.29 M glycine and 0.15% SDS.

After electrophoresis gels were subjected to silver staining. Low range, SDS PAGE molecular weight standards (Bio-rad) were used for comparison. The molecular weight markers were rabbit muscle phosphorylase b (97 400 Da), bovine serum albumin (66 200 Da), hen egg white ovalbumin (45 000 Da), bovine carbonic anhydrase (31 000 Da), soybean trypsin inhibitor (21 500 Da) and hen egg white lysozyme (14 400 Da). The stock solution of molecular weight standards was diluted 100 times for silver staining using sample buffer. The diluted standards were incubated for 5 min in a boiling water bath before use.

2.3.10.2 Non Denaturing PAGE for Endopeptidase Activity with Gelatin as the Substrate

Enzyme extracts from petals of fully open and senesced flowers had been used in this experiment. Electrophoresis was performed in a Studier-type gel using 7.5% (w/v) polyacrylamide gels with 0.1% (w/v) gelatin. The resolving gel was of a similar composition to the SDS PAGE except for polyacrylymide concentration, addition of 1 mL of 3% gelatin and the omission of SDS which was compensated for by the addition of extra water. Stacking gel comprised 3% (w/v) acrylamide monomers, buffered with 0.125 M Tris HCl buffer (pH 6.8), 0.05% (w/v) ammonium persulphate and 0.1% (w/v) TEMED. Electrophoresis buffer contained 37.5 mM Tris and 0.29 M glycine. Gels were run at 4°C for about 2-3 hr. The current was kept at 65 mA with unlimited voltage through the stacking gel and at 200 V through the separating gel. Sample for loading was prepared by mixing 2/3 volume of the protein extract with 1/3 volume of 60% (w/v) sucrose. Ten μL of 0.003% (w/v) bromophenol blue was loaded in a separate well as a tracking dye. After electrophoresis, gels were incubated and stained for endopeptidase activity as described in section 2.3.10.6.

2.3.10.3 Native Isoelectric Focusing (IEF) for Isozymes of LAP

This was carried out as described by Bollag, (1996) with slight modification to study the aminopeptidase isozymes in different plant parts and to study the effect of low-temperature storage of extracts on aminopeptidase isozymes of cv. Stella D'oro. Protein extracts used for IEF were concentrated in a dialysis tubing using 20,000-polyethylene glycol (PEG) (BDH) in a cold room at 4°C to concentrate the enzyme 7-10 fold. The concentrated enzyme was centrifuged for 10 min at 11,000 rpm at 4°C before subjecting the supernatant to IEF.

The gels (80 × 70 × 0.75 mm) were prepared in a Mini-Protean II gel system (Bio-rad, USA). The extracts were mixed with an equal volume of 2-fold native gel sample buffer and centrifuged for 5 min at 10,000 g before they were applied to the wells. The two-fold native gel sample buffer contained 60% glycerol and 4% ampholytes (in 1:5 ampholyte solutions (Sigma) of pH 3.5-10 and 4-6). Twenty mM NaOH was used as the catholyte in the upper buffer chamber and 10 mM phosphoric acid as anolyte in the lower buffer chamber. Both solutions were freshly prepared from 1 M stock solutions. Focusing conditions were 1.5 hr at 200 V and 1.5 hr at 400 V at 4°C. After electrophoresis, a strip of gel was cut out longitudinally for pI deterrmination and the rest of the gel was stained for aminopeptidase isozymes (section 2.3.10.7).

2.3.10.4 Coomassie Blue Staining Procedure

This was carried out after SDS PAGE of the partially purified enzyme extract to see the progress of enzyme purification. The gels were stained for 30 min at room temperature, with shaking at 20 rpm, in about 100 mL of Coomassie blue stain containing 50% absolute methanol, 10% glacial acetic acid (BDH) and 0.1% (w/v) Coomassie Brilliant Blue R 250 (Sigma). The staining solution was discarded and the gels were destained with 5% methanol and 10% glacial acetic acid at room temperature with shaking overnight or until the bands were clear, with several changes of destaining solution.

2.3.10.5 Silver Staining Procedure

The gels were washed in 50% methanol and 10% acetic acid for 30 min before being washed with distilled water 3 times each for 15 min. Then the gels were washed in 10% glutardialdehyde (BDH) for 30 min. After this, the gels were rinsed with 500 mL of distilled water for 30 min. This was repeated thrice. Then the gels were washed again with another 500 mL of distilled water overnight. On the next day, the gels

were soaked for 7 min in a freshly prepared solution of 30.6 mL 0.36% (w/v) NaOH, 2.06 mL NH_4OH, 6.0 mL of 0.2 g/mL $AgNO_3$ and 110 mL distilled water. Again the gels were washed with 4 changes of distilled water for 5 min each time. Then 250 mL of solution containing 2.5 mL of 100% ethanol, 625 µL of 1% citric acid (BDH), 62.5 µL of 38% formaldehyde (BDH) in distilled water were added to the gel. The gels were removed when the bands were dark enough and fixed in 5% acetic acid. All the steps were carried out with shaking at 20 rpm at room temperature.

2.3.10.6 Staining for Endopeptidase Activity on Gelatin

After electrophoresis gels were incubated at 37°C for 1 hr in 50 mM sodium acetate buffer (pH 4) (Appendix 2, Table D) with 2 mM cysteine and then stained for 30 min in 0.1% (w/v) amido black 10B (Sigma) (Guerrero et al., 1998) dissolved in 45% (v/v) methanol and 10% (v/v) acetic acid. The gels were destained in 7% acetic acid for 3 days or until clear bands appear on a dark blue background.

2.3.10.7 Staining for LAP Isozymes

After electrophoresis, gels were stained for aminopeptidase isozymes (Baes, 1973) as follows. Thirty mg of L-leucine-β(-naphthylamide hydrochloride) (Sigma) was first dissolved in 50% freshly prepared acetone before mixing with 100 mL of 0.1 M sodium phosphate buffer at pH 6 (Appendix 2, Table E). One hundred mg of Fast Garnet GBC salt (Sigma) was added into the solution, mixed and poured over the gel. The gel and the reaction mixture were incubated at 37°C for about 20 min for the development of red bands.

2.3.10.8 Determination of pI Value

A slice of about 0.5 cm from the unstained gel strip matching the position of LAP isozyme band in the stained gel was incubated in 1 mL of 10 mM KCl for 30 min with shaking at 20 rpm at room temperature. After this the pH of the KCl solution was determined using pH paper (Whatman, UK).

2.3.11 Partial Purification of Proteins

2.3.11.1 Ammonium Sulfate ($(NH_4)_2SO_4$) Precipitation

Proteins were extracted from 100 g of full bloom flower petals of cv. Stella Dóro collected on the day of opening in 148 mL of 0.01 M sodium phosphate buffer (pH 7) as described in section 2.3.2. $(NH_4)_2SO_4$ was added, a little at a time, to the crude protein extract in a cold room at 4°C by stirring to get to 30% (w/v) saturation. This was centrifuged for 15 min at 11,000 rpm at 4°C. The supernatant, was then brought to 60% saturation with 198 g/L $(NH_4)_2SO_4$, added a little at a time, at 4 °C. This was centrifuged for 15 min at 11,000 rpm at 4°C to obtain a pellet which was dissolved in 6 mL of sodium phosphate buffer (pH 7) and dialysed overnight with agitation at 4°C in 2 L of sodium phosphate buffer (pH 7). The dialysed extract was centrifuged at 11,000 rpm for 15 min at 4°C and then the supernatant was transferred to dialysis tubing and concentrated against 20,000-PEG in a cold room at 4°C to reduce the volume to about 3 mL.

2.3.11.2 Purifiaction by DEAE Cellulose Column

This step was carried out using a DEAE cellulose (Sigma) column (24 × 1.5 cm) attached to a peristaltic pump (Masterflex, Cole Parmer Instrument Co., USA, model

7014.20) and a fraction collector (Bio-rad, model 2110). The column was equillibriated with 300 mL of 0.01 M sodium phosphate buffer (pH 7) before 1.2 mL of 30-60% $(NH_4)_2SO_4$-precipitated proteins were loaded onto the column. After this, the column was washed with 45 mL of 0.01 M sodium phosphate buffer (pH 7) and 40 fractions of 1.5 mL each were collected. Then 0.4 M sodium chloride in 0.01 M phosphate buffer at pH 7 was added before 40 more fractions were collected. The absorbance of all fractions was determined at 280 nm and LAP activity in fractions 9 to 80 was assayed. The fractions with a LAP activity of at least 48 absorbance units per hr at 410 nm were pooled and concentrated against 20,000-PEG. This was used for the determination of the total protein content, LAP activity, endopeptidase activity and SDS PAGE analysis.

2.4 NUCLEIC ACIDS ASSOCIATED WITH DEVELOPMENT AND SENESCENCE OF DAYLILY FLOWERS

2.4.1 Bacterial Strain and *lap* cDNA

The plasmid containing PM 25 *lap* cDNA insert from *Arabidopsis thaliana* was provided by Dr. W. Weiler (Bartling and Weiler, 1992). *E. coli* strain HB101 (Maniatis et al., 1982) was used as the standard transformation host. Competent *E. coli* cells were prepared and transformed by the calcium chloride procedure (Maniatis et al., 1982).

2.4.2 Microbiological Media and Culture Conditions

The transformed bacterium as described in 2.4.1 was grown in Luria-Bertani medium (LB) (Maniatis et al., 1982) with 50 μg/mL of ampicillin (Sigma). LB medium containing 1% bacto tryptone (Difco, USA), 5% bacto yeast extract (Difco) and 1%

sodium chloride was autoclaved at 120°C and 147 kPa pressure for 15 min. The required volume of the ampicillin from a filter-sterilized (using a 0.22 μm filter of Millipore, Australia) stock solution of 100 mg/mL was added to the autoclaved medium just before use. If necessary, the medium was solidified with 1.2% (w/v) bacto-agar (Difco). When grown in broth cultures, single colony inoculations were placed into 10 mL of LB broth in Universal bottles and incubated overnight at 37°C with shaking at a rate of 20 rpm in the darkness.

2.4.3 Extraction of Plasmid cDNA from Bacteria by Alkaline-Lysis Method and Recovery of *lap* DNA from Low-Melting-Temperature Agarose

Standard minipreparations of plasmids were performed as detailed in Maniatis et al., (1982) with minor modifications. One mL of an overnight culture of bacteria in an Eppendorf tube was centrifuged for 1 min at 11,000 rpm at 4°C and the medium was removed by aspiration leaving the pellet as dry as possible. The pellet was resuspened by vortexing in 100 μL of ice cold solution of 50 mM glucose, 10 mM EDTA and 25 mM Tris HCl (pH 8.0) (Appendix 2, Table C3) and stored at room temperature for 5 min. Two hundred μL of a fresh solution of 0.2 M NaOH and 1% SDS were added. The contents were mixed by inverting the tube two or three times and stored for 5 min on ice. One hundred and fifty μL of cold potassium acetate solution (pH 4.8), made by mixing 60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid and 28.5 mL of distilled water was then added, vortexed for 10 sec, stored on ice for 5 min, and centrifuged for 5 min at 11,000 rpm at 4°C before the supernatant was transferred to a fresh tube. An equal volume of Tris-saturated phenol (Sigma):chloroform (BDH):isoamyl alcohol (BDH) in 25:24:1 was added, vortexed, centrifuged for 2 min and the supernatant was transferred to a fresh tube. Two volumes of absolute ethanol were added at room temperature, vortexed and left for 2 min, centrifuged for 5 min to precipitate plasmid DNA. The pellet was washed in 1 mL of 70% (v/v) ethanol and

dried briefly in a vacuum desiccator. The pellet was suspended in 50 μL of sterile distilled water and stored at -20°C.

The plasmid DNA (5 μg) was cut with *EcoRI* enzyme for 2 hr in a standard restriction enzyme digestion procedure (Maniatis et al., 1982). Agarose gel electrophoresis and subsequent staining of cut and uncut plasmid DNA and DNA markers were carried out using low-melting-temperature agarose at 1% as described in Maniatis et al., (1982).

2.4.4 Isolation of Plant Nucleic acids

2.4.4.1 Isolation of RNA from Plant Tissues

RNA was isolated from petals of cv. Stella D'oro. One hundred mg of petals were homogenized in 1 mL Trizol reagent (GibcoBrl, Australia) using a mortar and pestle and incubated for 5 min at 15-30°C in an Eppendorf tube. Then, 0.2 mL of chloroform was added, mixed vigorously for 15 sec, incubated for 2-3 min at room temperature and centrifuged for 15 min at 15,000 rpm at 4°C. The colourless aqueous phase was transferred into a new Eppendorf tube, and incubated for 10 min at room temperature with 0.5 mL of isopropanol. RNA was precipitated by centrifuging again for 15 min at 15,000 rpm at 4°C. The RNA pellet was washed in 1 mL of 75% (v/v) ethanol and stored at -80°C in another 1 mL of 75% ethanol.

Just before use, RNA pellet was precipitated again by centrifugation and dissolved in 1 mL of RNAase free sterile distilled water. RNAase free sterile distilled water was prepared by treating distilled water with 0.01% diethylpyrocarbonate (BDH) for 24 hr at 37°C before being autoclaved.

2.4.4.2 Estimation of Plant Nucleic Acid Concentration and Testing for Purity

Quantitation of nucleic acids was carried out by diluting 5 μL of daylily petal DNA or RNA preparation in 1 mL of sterile distilled water (for RNA used RNAse free water) and measuring the UV absorption at 260 and 280 nm using a SmartSpec™ 3000 spectrophotometer (Bio-rad). The nucleic acid solution was considered to be pure enough if the ratio of the absorbency at 260 nm/280 nm was greater than 1.8 and 2 for DNA and RNA respectively. The concentration of the RNA and DNA was assumed to be 40 μg/mL and 50 μg/mL respectively for every absorbance unit at 260 nm (Maniatis et al., 1982).

2.4.5 Hybridization of RNA and DNA

DNA probe (2.4.5.1) labelling and its subsequent hybridization with daylily petal RNA had been carried out according to the instruction manual using DIG DNA labelling and detection kit (Boehringer Mannheim) with slight modification.

2.4.5.1 DNA Probe Preparation

The template used for digoxigenin labelling was the *lap* cDNA extracted from low-melting agarose gel. The template DNA (0.5-3 μg) was diluted to a total volume of 15 μL in an Eppendof tube, denatured by heating for 10 min in a boiling water bath and immediately cooled on ice. The *lap* cDNA was incubated overnight at 37°C in 2 μL of hexanucleotide mix, 2 μL dNTP mixture and 1 μL of Klenow enzyme. The reaction was stopped by adding 2 μL of 0.2 M EDTA (pH 8.0) and the labelled DNA was precipitated by adding 2.5 μL of 4 M lithium chloride (Sigma) and 75 μL of prechilled (-20°C) 100% ethanol. The mixture was incubated for 30 min at -80°C

before centrifuging for 15 min at 11,000 rpm. The pellet was washed with 50 μL of 70% cold ethanol and dissolved in 20 μL of sterile distilled water.

2.4.5.2 Estimation of Labelled *lap* Probe Concentration

A dilution series of labelled *lap* probe and DIG-labelled control-DNA of known concentration provided in the kit were prepared and spotted on positively charged nylon membrane (Boehringer Mannheim). After following the detection protocol (2.4.3.6) the colour intensities and diameters of spots of known concentrations were compared to estimate the concentration of the probe.

2.4.5.3 Template Preparation for Dot Blots

The template *lap* DNA probe (used for estimation of concentration) or RNA was denatured by heating in a boiling water bath for 5 min and quickly chilling on ice. The required volume (1-5 μL) of DNA probe or RNA was spotted onto a positively charged nylon membrane (Boehringer Mannheim) and cross linked by exposure to UV light (254 nm, 120,000 μJ; UVC-515, SciTech, New Zealand) for 5 min.

2.4.5.4 DNA and RNA Dot Blot Hybridization

DNA dot blot hybridization was carried out using standard hybridization buffer that contained 5 × SSC, 0.1% (w/v) N-lauroylsarcosine (Sigma), 0.02% (w/v) SDS, and 1% blocking solution at 50°C in a 20 mL-capped-glass vial. Blocking reagent was prepared as a 10 × stock solution using the blocking powder provided in the kit. The membrane with UV-cross linked daylily genomic DNA was incubated in prewarmed (at 50°C) standard hybridization buffer (20 ml/100 cm^2 of membrane) in a

hybridization tube (4 × 30 cm) (Hybaid, SciTech, New Zealand) for 30 min for prehybridization. The probe was denatured by boiling for 5 min in a boiling water bath and rapidly cooling on ice. The probe was added to standard hybridization buffer (2.5 mL/100 cm^2 of membrane) and incubated at 50°C. The prehybridization solution was poured off and added the probe-buffer mixture before hybridising for 48 hr at 50°C and 20 rpm.

RNA hybridization was carried out in the same way but using "high SDS" hybridization buffer instead of standard hybridization buffer. "High SDS" hybridization buffer contained 7% SDS, 50% formamide, 5 × SSC, 0.1% (w/v) N-lauroylsarcosine, and 2% blocking solution. All the reagents that were used for RNA dot blot hybridization had been treated with 0.01% diethylepyrocarbonate to remove RNAases. Glassware and other equipment were treated with 0.5 M NaOH for 15 min to remove RNAase, rinsed thoroughly in distilled water and autoclaved before using.

2.4.5.5 Post-Hybridization Washes

After hybridization with the *lap* DNA probe, the membrane was washed twice in a large volume (15-20 mL) of 2 × SSC and 1% SDS for 17 min at room temperature and twice in 0.1 × SSC and 0.1% SDS (15-20 mL) for 7 min at 50°C. Membranes subjected to RNA dot blot hybridization were washed twice in 15-20 mL of 2 × SSC and 1% SDS for 7 min at room temperature and twice in 15-20 mL 0.1 × SSC and 0.1% SDS for 17 min at 50°C. Post-hybridization washes were carried out in 50 mL-capped-plastic jars under constant agitation.

2.4.5.6.Detection Procedure

After post-hybridisation washes the membrane was rinsed for 5 min in washing buffer containing 3% Tween 20 (v/v) (BDH) in maleic acid buffer (0.1 M maleic acid, 0.15 M sodium chloride; pH 7.5). Then the membrane was incubated for 30 min in 1 × blocking solution (100 mL/100 cm^2 of membrane) and then for another 30 min in antibody solution (20 mL/100 cm^2 of membrane). The antibody solution was prepared by diluting anti-DIG-AP conjugate provided in the kit in 1 × blocking solution (1:10000). Afterwards the membrane was washed twice in washing buffer (100 mL/100 cm^2 of membrane) for 15 min each and equilibrated for 5 min in detection buffer (20 mL/100 cm^2 of membrane) consisting of 0.1 M Tris HCl (Appendix 2, Table C3), 0.1 M sodium chloride and 50 mM magnesium chloride (Sigma) at pH 9.5. Then the membrane was incubated in freshly prepared colour substrate solution containing (0.5 mL) 200 µL NBT/BCIP stock solution provided in the kit in 10 mL of detection buffer at 37°C in darkness until the spots or bands appeared. Just before the backgroun colour appeared, the membrane was washed in water and photographed.

2.5 DATA HANDLING

The Microsoft Office 2000, Excel 2000 was used for the analysis of all the data and, two factor ANOVA for the experiment on endogenous proteolytic inhibitors and promoters.

CHAPTER 3

RESULTS

3.1 MICROPROPAGATION

3.1.1. Initiation of Shoot Primordia-like Structures from Floral Explants

The establishment of four daylily cultivars into sterile culture was achieved using petal and ovary explants from small- and medium-sized buds. The effect of contamination on culture efficiency was minimal, suggesting the sterilisation protocol was satisfactory. Explants were grown for 2-4 months in 1/2 strength MS containing plant growth regulators. All the explants increased in size once they were introduced into media containing plant growth regulators. Of them, some showed callus like development and eventually produced tiny shoot primordia-like structures (Plate 3.1A) which would develop into shoots later. Other explants remained alive for about 2-3 months but eventually dried or turned brown and died. The percentages of explants that produced shoot primordia-like structures 2 months after growing in plant growth regulator-containing media are shown in Tables 3.1-3.4 and examined more closely below.

In the cv. Stella D'oro, ovary explants from medium-sized buds responded best to media containing either the combination of 5 mg/L NAA and 1 mg/L 2iP (Table 3.1A and B) or that of 1 mg/L NAA and 5 mg/L BA, whereas those from small buds responded best to media containing 3 mg/L each of NAA and BA. On the other hand, up to 25% of inner and outer petal explants from medium-sized buds formed shoot primordia-like structures in all the growth regulator combinations tested. Outer petal explants from small buds showed a similar response to all media

tested except that with 1 mg/L NAA and 5 mg/L BA which did not support shoot bud formation. Up to about 40% of inner petal explants from small buds produced primordia-like structures except in media containing either the combination of 5 mg/L NAA and 1 mg/L 2iP or that of 1 mg/L NAA and 5 mg/L 2iP which did not induce any shoot primordia development.

In the cv. Siloam Ribbon Candy, explants from ovaries of both medium- and small-sized buds were highly responsive in forming shoot primordia-like structures, to all the plant growth regulator-combinations tested except those of medium-sized buds in 1mg/L NAA with 5 mg/L 2iP (Tables 3.2A). Outer petal explants from medium-sized buds placed in medium with 5 mg/L NAA and 1 mg/L BA did not show shoot primordia-like development. Up to 35% of all other explants from outer and inner petals of buds of both sizes developed shoot primordia-like structures (Tables 3.2A and B).

In the cv. Chorus Line, petal and ovary explants from medium-sized buds did not produce shoot primordia-like structures in response to the combination of 3 mg/L each of NAA and 2iP (Table 3.3A). Up to 43% of petal explants from medium-sized buds in all other media combinations produced shoot primordia-like structures. A response of 63% has been achieved by outer petals from small buds in 3 mg/L each of NAA and BA (Table 3.3B). Up to 32% outer and inner petals from small buds formed shoot primordia-like structures in response to other combinations of growth regulators. A very high response (between 76-100%) was achieved by ovary explants from medium-sized and small-sized buds in some growth regulator combinations tested (Tables 3.3A and B).

A

B C

E

F

D

Plate 3.1
Micropropagation of daylily cv. Stella D'oro by tissue culture. A: Shoot primordia-like structures developed from outer petal explants; B & C: Plantlets in subculture; C: Plantlet ready to be transferred to soil; D: Tissue cultured plants in soil. E: Young flower scape from a tissue cultured plant; F: Flower from tissue cultured plant. (A, B and C are representative of stages of micropropagation of other cultivars as well).

Table 3.1: Proportions of explants of buds of the cv. Stella D'oro that showed shoot primordia-like development after 2 months on ½ strength MS containing plant growth regulators. For medium-sized buds, the number of each type of explants used in a treatment was as follows: 33 outer petal, 27 inner petal and 6 ovary explants. For small-sized buds, the number of each type of explants used in a treatment was as follows: 24 outer petal, 18 inner petal and 6 ovary explants.

A

Medium	Explants from Outer Petals	Explants from Inner Petals	Explants from Ovary
5 mg/L NAA and 1 mg/L 2iP	+ (24)	+ (12)	++ (50)
3 mg/L NAA and 3 mg/L 2iP	+ (9)	+ (3)	- (0)
1 mg/L NAA and 5 mg/L 2iP	+ (9)	+ (6)	- (0)
5 mg/L NAA and 1 mg/L BA	+ (15)	+ (19)	- (0)
3 mg/L NAA and 3 mg/L BA	+ (12)	+ (3)	- (0)
1 mg/L NAA and 5 mg/L BA	+ (2)	+ (3)	+ (17)

B

Medium	Explants from Outer Petals	Explants from Inner Petals	Explants from Ovary
5 mg/L NAA and 1 mg/L 2iP	+ (17)	- (0)	- (0)
3 mg/L NAA and 3 mg/L 2iP	+ (15)	++ (33)	- (0)
1 mg/L NAA and 5 mg/L 2iP	+ (8)	- (0)	- (0)
5 mg/L NAA and 1 mg/L BA	+ (22)	++ (38)	- (0)
3 mg/L NAA and 3 mg/L BA	+ (13)	++ (35)	+++ (67)
1 mg/L NAA and 5 mg/L BA	- (0)	+ (11)	- (0)

A: medium-sized (3.5 cm long) bud; B: small-sized (2 cm long) bud.

- : no shoot primordia-like structures;

+ : 1-25% of the explants showed shoot primordia-like structures;

++ : 26-50% of the explants showed shoot primordia-like structures;

+++ : 51-75% of the explants showed shoot primordia-like structures;

++++ : 76-100% of the explants showed shoot primordia-like structures.

Values in the brackets indicate the actual percentage.

Table 3.2: Proportions of explants of buds of the cv. Siloam Ribbon Candy that showed shoot primordia-like development after 2 months on ½ strength MS containing plant growth regulators. For medium-sized buds, the number of each type of explants used in a treatment was as follows: 33 outer petal, 27 inner petal and 6 ovary explants. For small-sized buds, the number of each type of explants used in a treatment was as follows: 24 outer petal, 18 inner petal and 6 ovary explants.

A

Medium	Explants from Outer Petals	Explants from Inner Petals	Explants from Ovary
5 mg/L NAA and 1 mg/L 2iP	+ (7)	+ (8)	++++ (83)
3 mg/L NAA and 3 mg/L 2iP	++ (29)	+ (21)	++++ (100)
1 mg/L NAA and 5 mg/L 2iP	+ (18)	+ (20)	++ (33)
5 mg/L NAA and 1 mg/L BA	- (0)	+ (11)	++++ (83)
3 mg/L NAA and 3 mg/L BA	++ (35)	+ (6)	++++ (83)
1 mg/L NAA and 5 mg/L BA	++ (30)	+ (6)	++++ (80)

B

Medium	Explants from Outer Petals	Explants from Inner Petals	Explants from Ovary
5 mg/L NAA and 1 mg/L 2iP	+ (21)	+ (7)	++++ (83)
3 mg/L NAA and 3 mg/L 2iP	++ (26)	+ (15)	++++ (100)
1 mg/L NAA and 5 mg/L 2iP	+ (17)	+ (20)	++++ (100)
5 mg/L NAA and 1 mg/L BA	++ (35)	+ (8)	++++ (90)
3 mg/L NAA and 3 mg/L BA	++ (42)	+ (7)	++++ (100)
1 mg/L NAA and 5 mg/L BA	++ (31)	++ (27)	++++ (100)

A: medium-sized (4 cm long) bud; B: small-sized (2 cm long) bud.

- : no shoot primordia-like structures;

+ : 1-25% of the explants showed shoot primordia-like structures;

++ : 26-50% of the explants showed shoot primordia-like structures;

+++ : 51-75% of the explants showed shoot primordia-like structures;

++++ : 76-100% of the explants showed shoot primordia-like structures.

Values in the brackets indicate the actual percentage.

Table 3.3: Proportions of explants of buds of the cv. Chorus Line that showed shoot primordia-like development after 2 months on ½ strength MS containing plant growth regulators. For medium-sized buds, the number of each type of explants used in a treatment was as follows: 33 outer petal, 27 inner petal and 6 ovary explants. For small-sized buds, the number of each type of explants used in a treatment was as follows: 24 outer petal, 18 inner petal and 6 ovary explants.

A

Medium	Explants from Outer Petals	Explants from Inner Petals	Explants from Ovary
5 mg/L NAA and 1 mg/L 2iP	+ (10)	+ (2)	++ (33)
3 mg/L NAA and 3 mg/L 2iP	- (0)	- (0)	- (0)
1 mg/L NAA and 5 mg/L 2iP	+ (10)	+ (16)	++++ (83)
5 mg/L NAA and 1 mg/L BA	++ (43)	++ (32)	++++ (83)
3 mg/L NAA and 3 mg/L BA	+ (11)	+ (8)	++ (50)
1 mg/L NAA and 5 mg/L BA	++ (31)	++ (21)	++++ (100)

B

Medium	Explants from Outer Petals	Explants from Inner Petals	Explants from Ovary
5 mg/L NAA and 1 mg/L 2iP	+ (9)	+ (4)	++ (33)
3 mg/L NAA and 3 mg/L 2iP	+ (7)	+ (12)	++ (12)
1 mg/L NAA and 5 mg/L 2iP	+ (17)	+ (9)	++++ (83)
5 mg/L NAA and 1 mg/L BA	++ (43)	++ (32)	+++ (72)
3 mg/L NAA and 3 mg/L BA	+++ (63)	+ (24)	+++ (75)
1 mg/L NAA and 5 mg/L BA	+ (21)	+ (19)	++ (50)

A: medium-sized (3.5 cm long) bud; B: small-sized (2 cm long) buds.

- : no shoot primordia-like structures;

+ : 1-25% of the explants showed shoot primordia-like structures;

++ : 26-50% of the explants showed shoot primordia-like structures;

+++ : 51-75% of the explants showed shoot primordia-like structures;

++++ : 76-100% of the explants showed shoot primordia-like structures.

Values in the brackets indicate the actual percentage.

About 1-20% of outer and inner petal explants from both medium- and small-sized buds of cv. Dallas Star formed shoot primordia-like structures in all combinations of growth regulators tested except in 2 combinations (Tables 3.4A and B). Inner petal explants from medium-sized buds cultured on the medium containing 1 mg/L NAA and 5 mg/L 2iP and outer petal explants from small-sized buds cultured on the medium containing 1 mg/L NAA and 5 mg/L BA did not produce any shoot primordia-like structures. Similarly, ovary explants from medium-sized buds cultured on media containing either the combination of 5 mg/L NAA and 1 mg/L BA, or that of 3 mg/L each of NAA and BA did not produce shoot primordia-like structures. In other media however, ovary explants from medium-sized buds formed shoot primordia-like structures with the best response to that with 5 mg/L NAA and 1 mg/L 2iP. At least 30% of ovary explants from small buds in all combinations of growth regulators produced shoot primordia-like structures.

Overall, the size of the explant did not have an effect on initiation of shoots except in the case of ovary explants of cv. Dallas Star, where medium-sized buds resulted in a poorer response than small-sized ones.

3.1.2 Effect of Position of the Petal Explant on Initiation of Shoot Primordia-like Structures

Cultivar Stella D'oro had been used to test whether the position in the petals from which the explant was obtained had any effect on the formation of shoot primordia-like structures. It was found that all the petal explants that gave rise to shoot primordia-like structures originally came from the base of the petals or from the tubes. Explants from the middle or the tip of the petals did not produce shoot primordia-like structures at all.

Table 3.4: Proportions of explants of buds of the cv. Dallas Star that showed shoot primordia-like development after 2 months on ½ strength MS containing plant growth regulators. For medium-sized buds, the number of each type of explants used in a treatment was as follows: 33 outer petal, 27 inner petal and 6 ovary explants. For small-sized buds, the number of each type of explants used in a treatment was as follows: 24 outer petal, 18 inner petal and 6 ovary explants.

A

Medium	Explants from Outer Petals	Explants from Inner Petals	Explants from Ovary
5 mg/L NAA and 1 mg/L 2iP	+ (3)	+ (9)	+++ (67)
3 mg/L NAA and 3 mg/L 2iP	+ (7)	+ (5)	++ (33)
1 mg/L NAA and 5 mg/L 2iP	+ (7)	- (0)	+ (17)
5 mg/L NAA and 1 mg/L BA	+ (5)	+ (3)	- (0)
3 mg/L NAA and 3 mg/L BA	+ (12)	+ (11)	- (0)
1 mg/L NAA and 5 mg/L BA	+ (3)	+ (2)	+ (17)

B

Medium	Explants from Outer Petals	Explants from Inner Petals	Explants from Ovary
5 mg/L NAA and 1 mg/L 2iP	+ (9)	+ (18)	++ (33)
3 mg/L NAA and 3 mg/L 2iP	+ (21)	+ (3)	++++ (83)
1 mg/L NAA and 5 mg/L 2iP	+ (13)	+ (9)	++ (30)
5 mg/L NAA and 1 mg/L BA	+ (17)	+ (3)	+++ (57)
3 mg/L NAA and 3 mg/L BA	+ (6)	+ (7)	++ (43)
1 mg/L NAA and 5 mg/L BA	- (0)	+ (7)	++ (33)

A: medium-sized (5 cm long) bud; B: small-sized (3 cm long) bud.

- : no shoot primordia-like structures;

+ : 1-25% of the explants showed shoot primordia-like structures;

++ : 26-50% of the explants showed shoot primordia-like structures;

+++ : 51-75% of the explants showed shoot primordia-like structures;

++++ : 76-100% of the explants showed shoot primordia-like structures.

Values in the brackets indicate the actual percentage.

3.1.3 Regeneration of Plantlets from Shoot Primordia-like Structures

Once shoot primordia-like structures had been generated in growth regulator-containing media, they were all transferred and subcultured in growth regulator free-1/2 strength MS to see if they would develop into shoots and then initiate roots, i.e. regenerate of complete plantlets.

Production of a large number of shoot primordia-like structures by a particular explant in a particular combination of plant growth regulators does not necessarily guarantee that they will develop into plantlets in the rooting medium. Some of the shoot primordia-like structures died or got contaminated while being cultured in growth regulator free- ½ strength MS. The number of plantlets obtained from each explant type, 8 months after the initial introduction into media containing different plant growth regulators are shown in the Tables 3.5-3.8.

Outer and inner petal explants from medium-sized buds of cv. Stella D'oro produced many plantlets upon transfer to growth regulator-free medium from (Plate 3.1B and C) medium containing 3 mg/L NAA and 3 mg/L BA (Table 3.5A), while ovary explants of the same type of buds gave many plantlets after they were transferred from medium with 1 mg/L NAA and 5 mg/L BA. Similarly, inner petal and ovary explants from small buds in medium containing 3 mg/L NAA and 3 mg/L BA also produced many plantlets (Table 3.5B). In many other explant/growth regulator combinations, plantlet production was between 0 and 2.

In the cv. Siloam Ribbon Candy, ovary explants first grown in medium containing the combination of 5 mg/L NAA and 1 mg/L BA, or 3 mg/L NAA and 3 mg/L BA from both small- and medium-sized buds produced many plantlets (Table 3.6A and B). Ovary explants from small buds grown in medium containing 1 mg/L NAA and

5 mg/L BA gave rise to about 14 plantlets. In all other cases, the numbers of plantlets produced were between 0-5.

In the cv. Chorus Line, outer petal explants from medium-sized buds initially grown in medium containing 5 mg/L NAA and 1 mg/L BA produced many plantlets (Table 3.7A). Outer petal and ovary explants from small buds grown in 1 mg/L NAA and 5 mg/L BA also gave rise to many plantlets (Table 3.7B). In other combinations of explants and growth regulators, plantlet regeneration was negligible.

Plantlet production by cv. Dallas Star from both medium-sized and small buds is very poor when compared with other cultivars (Table 3.8A and B). Outer petal explants from medium-sized buds grown in 3 mg/L NAA and 3 mg/L BA produced 3 plantlets while ovary explants from small-sized buds grown in 3 mg/L NAA and 3 mg/L 2iP produced one plantlet. All other explants in all other growth regulator combinations did not produce plantlets.

3.1.4 Effect of Sucrose on Root Formation in Shoot Cultures of cv. Stella D'oro

Effect of sucrose concentration on the induction of roots was tested by introducing rootless shoots into growth regulator free-1/2 strength MS medium containing 0, 3 and 6% sucrose. Out of 6 replicates in each medium 4, 5 and 6 shoots produced 1 root each in 7 days. After 14 days all the shoots had produced one root each in all the sucrose concentrations tested. All the roots appeared the same, with same thickness and were pale yellow in colour.

Table 3.5: Number of plantlets obtained from buds of the cv. Stella D'oro, 8 months after the initial introduction of explants into media containing plant growth regulators.

A

Medium	Explants from Outer Petals	Explants from Inner Petals	Explants from Ovary
5 mg/L NAA and 1 mg/L 2iP	[0-1] *1	[0-1] *1	[0]
3 mg/L NAA and 3 mg/L 2iP	[0]	[0]	#[0]
1 mg/L NAA and 5 mg/L 2iP	[0-1] *1	[0]	#[0]
5 mg/L NAA and 1 mg/L BA	[0-1] *1	[0-1] *2	#[0]
3 mg/L NAA and 3 mg/L BA	[5-10] *many	[1-5] *many	#[0]
1 mg/L NAA and 5 mg/L BA	[0-2] *2	[0-1] *1	[4-20] *many

B

Medium	Explants from Outer Petals	Inner Petals	Ovary
5 mg/L NAA and 1 mg/L 2iP	[0]	#[0]	#[0]
3 mg/L NAA and 3 mg/L 2iP	[0]	[0-1] *1	#[0]
1 mg/L NAA and 5 mg/L 2iP	[0-8] *8	#[0]	#[0]
5 mg/L NAA and 1 mg/L BA	[0-9] *10	[0]	#[0]
3 mg/L NAA and 3 mg/L BA	[0-1] *1	[0-10] *many	[0-12] *many
1 mg/L NAA and 5 mg/L BA	#[0-1] *1	[0]	#[0]

A: medium-sized (3.5 cm long) bud; B: small-sized (2 cm long) bud.

Plantlets were regenerated in the growth regulator-free ½ strength MS medium. [ ] denotes the minimum to maximum number of plantlets regenerated per explant. * denotes the total number of plantlets obtained from all the explants that produced shoot primordia-like structures; many: >20 plantlets. At least 1 explant from ovary and 10 explants from outer and inner petals, were transferred to growth regulator-free ½ strength MS. # indicates that no explants were transferred to growth regulator-free medium due to lack of initiation of shoot primordia-like structures.

Table 3.6: Number of plantlets obtained from buds of the cv. Siloam Ribbon Candy, 8 months after the initial introduction of explants into media containing plant growth regulators.

A

Medium	Explants from Outer Petals	Explants from Inner Petals	Explants from Ovary
5 mg/L NAA and 1 mg/L 2iP	[0]	[0]	[0]
3 mg/L NAA and 3 mg/L 2iP	[0-1] *1	[0]	[0-1] *1
1 mg/L NAA and 5 mg/L 2iP	[0]	[0-2] *2	[0]
5 mg/L NAA and 1 mg/L BA	#[0]	[0]	[1-12] *many
3 mg/L NAA and 3 mg/L BA	[0-2] *2	[0]	[2->20] *many
1 mg/L NAA and 5 mg/L BA	[0-3] *4	[0]	[0-4] *4

B

Medium	Explants from Outer Petals	Explants from Inner Petals	Explants from Ovary
5 mg/L NAA and 1 mg/L 2iP	[0]	[0]	[0-3] *3
3 mg/L NAA and 3 mg/L 2iP	[0]	[0]	[0-3] *7
1 mg/L NAA and 5 mg/L 2iP	[0]	[0]	[0]
5 mg/L NAA and 1 mg/L BA	[0-2] *5	[0]	[3-12] *many
3 mg/L NAA and 3 mg/L BA	[0-2] *3	[0]	[1->20] *many
1 mg/L NAA and 5 mg/L BA	[0-1] *1	[0]	[1-3] *14

A: medium-sized (4 cm long) bud; B: small-sized (2 cm long) bud.

Plantlets were regenerated in the growth regulator-free ½ strength MS medium. [ ] denotes the minimum to maximum number of plantlets regenerated per explant. * denotes the total number of plantlets obtained from all the explants that produced shoot primordia-like structures; many: >20 plantlets. At least 1 explant from ovary and 10 explants from outer and inner petals, were transferred to growth regulator-free ½ strength MS. # indicates that no explants were transferred to growth regulator-free medium due to lack of initiation of shoot primordia-like structures.

Table 3.7: Number of plantlets obtained from buds of the cv. Chorus Line, 8 months after the initial introduction of explants into media containing plant growth regulators.

A

Medium	Explants from Outer Petals	Explants from Inner Petals	Explants from Ovary
5 mg/L NAA and 1 mg/L 2iP	[0]	[0]	[0]
3 mg/L NAA and 3 mg/L 2iP	#[0]	#[0]	#[0]
1 mg/L NAA and 5 mg/L 2iP	[0]	[0-2] *2	[0]
5 mg/L NAA and 1 mg/L BA	[0-10] *many	[0-1] *1	[0-1] *1
3 mg/L NAA and 3 mg/L BA	[0-6] *9	[0-2] *2	[0]
1 mg/L NAA and 5 mg/L BA	[0-6] *12	[0-2] *2	[0]

B

Medium	Explants from Outer Petals	Explants from Inner Petals	Explants from Ovary
5 mg/L NAA and 1 mg/L 2iP	[0]	[0]	[0]
3 mg/L NAA and 3 mg/L 2iP	[0]	[0]	[0]
1 mg/L NAA and 5 mg/L 2iP	[0]	[0]	[0]
5 mg/L NAA and 1 mg/L BA	[0-1] *2	[0]	[0]
3 mg/L NAA and 3 mg/L BA	[0-4] *4	[0]	[0-1] *1
1 mg/L NAA and 5 mg/L BA	[0-6] *many	[0-2] *4	[5-12] *many

A: medium-sized (3.5 cm long) bud; B: small-sized (2 cm long) bud.

Plantlets were regenerated in the growth regulator free-1/2 strength MS medium. [ ] denotes the minimum to maximum number of plantlets regenerated per explant. * denotes the total number of plantlets obtained from all the explants that produced shoot primordia-like structures; many: >20 plantlets. At least 1 explant from ovary and 10 explants from outer and inner petals were transferred to growth regulator-free ½ strength MS. # indicates that no explants were transferred to growth regulator-free medium due to lack of initiation of shoot primordia-like structures.

Table 3.8: Number of plantlets obtained from buds of the cv. Dallas Star, 8 months after the initial introduction of explants into media containing plant growth regulators.

A

Medium	Explants from Outer Petals	Explants from Inner Petals	Explants from Ovary
5 mg/L NAA and 1 mg/L 2iP	[0]	[0]	[0]
3 mg/L NAA and 3 mg/L 2iP	[0]	[0]	[0]
1 mg/L NAA and 5 mg/L 2iP	[0]	#[0]	[0]
5 mg/L NAA and 1 mg/L BA	[0]	[0]	#[0]
3 mg/L NAA and 3 mg/L BA	[0-3] *3	[0]	#[0]
1 mg/L NAA and 5 mg/L BA	[0]	[0]	[0]

B

Medium	Explants from Outer Petals	Explants from Inner Petals	Explants from Ovary
5 mg/L NAA and 1 mg/L 2iP	[0]	[0]	[0]
3 mg/L NAA and 3 mg/L 2iP	[0]	[0]	[0-1] *1
1 mg/L NAA and 5 mg/L 2iP	[0]	[0]	[0]
5 mg/L NAA and 1 mg/L BA	[0]	[0]	[0]
3 mg/L NAA and 3 mg/L BA	[0]	[0]	[0]
1 mg/L NAA and 5 mg/L BA	#[0]	[0]	[0]

A: medium-sized (5 cm long) bud; B: small-sized (3 cm long) bud. Plantlets were regenerated in the growth regulator free-1/2 strength MS medium. [ ] denotes the minimum to maximum number of plantlets regenerated per explant. * denotes the total number of plantlets obtained from all the explants that produced shoot primordia-like structures; many: >20 plantlets. At least 1 explant from ovary and 10 explants from outer and inner petals were transferred to growth regulator-free ½ strength MS. # indicates that no explants were transferred to growth regulator-free medium due to lack of initiation of shoot primordia-like structures.

3.1.5 Establishment of Aseptically Grown Plants in Soil and production of Flowers of cv. Stella D'oro

In both trials, all the plantlets transferred to soil (Plate 3.1D) survived. These plants matured and produced first flowers (Plate 3.1E and F) 6 months after transferring to soil. Thereafter, they continued to flower every summer. The plants and flowers were morphologically similar to the parent plants from which the explants were obtained. Plants tested in different sucrose concentrations for rooting also showed similar behaviour to the other tissue-cultured plants.

3.1.6 Use of Tissue Cultured Plant Material in Experiments on Flower Senescence

Of the four cultivars, micropropagation of cv. Stella D'oro was attempted in this research. Hence, it was the first to produce plantlets which were the only ones that were transferred to soil. A large number of flowers of cv. Stella D'oro were produced once the plantlets were transferred to soil. For this reason, most of the experiments on flower senescence were carried out using flowers of this cultivar.

3.2 DEVELOPMENT OF DAYLILY FLOWERS

3.2.1 Morphological Changes

A study on the natural development and senescence pattern of daylily flowers of six cultivars showed that there are three major developmental stages, namely bud, full bloom and 'senesced' (wilted) stages (Plate 3.2). A closer examination of the development of the flowers of cv. Stella D'oro was carried out (Plate 3.3). The following is a general description of the morphological changes accompanying the

development of immature daylily floral buds and the following life span of the flowers mainly based on the observations of cv. Stella D'oro. As the flower buds enlarge in size their colour begin to change from green to the final colouration of the flower (yellow in the case of cv. Stella D'oro). Around 9 a.m. on the day before opening, the buds are almost in maximum size and have tightly closed or slightly open sepals, with or without the stigma protruding. Flowers are half open and have dehisced anthers by about 9 p.m. on the same day. At 9 a.m. the next day, the flowers are fully open and are in the form of typical lily shape flowers. The first symptoms of senescence, the appearance of translucent areas along the margins of the inner petals can be observed 12 hours later. By this time flowers have begun to close. Translucent areas enlarge and combine. With time, outer petal margins also become translucent, and the flowers are fully wilted the next morning. In fully senesced flowers the inner petals lose firmness and become watery-collapsing bundles while outer petals become slightly dry. At this stage, petals of cvs. Siloam Ribbon Candy, Chorus Line and Dallas Star exude a reddish pigment. The next day, i.e. 48 h after the fully open stage the senesced flowers are dried further. About 4-5 days after opening dried flowers fall from the plant. If the ovules are fertilized, the remaining ovary being developed into a fruit can be seen on the flower stalk.

3.2.2 Change in Fresh Weight during Flower Development

Change in fresh weight during the development and senescence of flowers of six daylily cultivars was determined by weighing buds and flowers collected at different stages (Figure 3.1). Fresh weight changes appear to follow a general pattern during flower development and senescence in all the cultivars tested except in cv. So Sweet. In general as buds developed the fresh weight of daylily flowers reached a maximum at the fully open stage (0 h) and then decreased as the flower wilted ('senesced' stages at 24 h and 48 h after full bloom stage). In the cv. So Sweet, the fresh weight increased during bud opening. But after the flower was

fully open, its fresh weight increased further and reached a maximum 24 h later. After this it decreased.

3.2.3. Changes in Proteins during Flower Development

3.2.3.1 Soluble Proteins

There was a change in soluble protein content in daylily flowers during flower development and senescence (Table 3.9). An increase in soluble protein content during flower opening had been observed at least in the cultivars Stella D'oro and Chorus Line. In the cv. Dallas Star the soluble protein content seemed to remain unchanged during bud opening. In all the three cultivars tested there was a large reduction in the soluble protein content during flower senescence. Analysis by SDS PAGE indicates that this change in protein content was associated with loss of certain proteins as well as synthesis of new proteins (Plate 3.4).

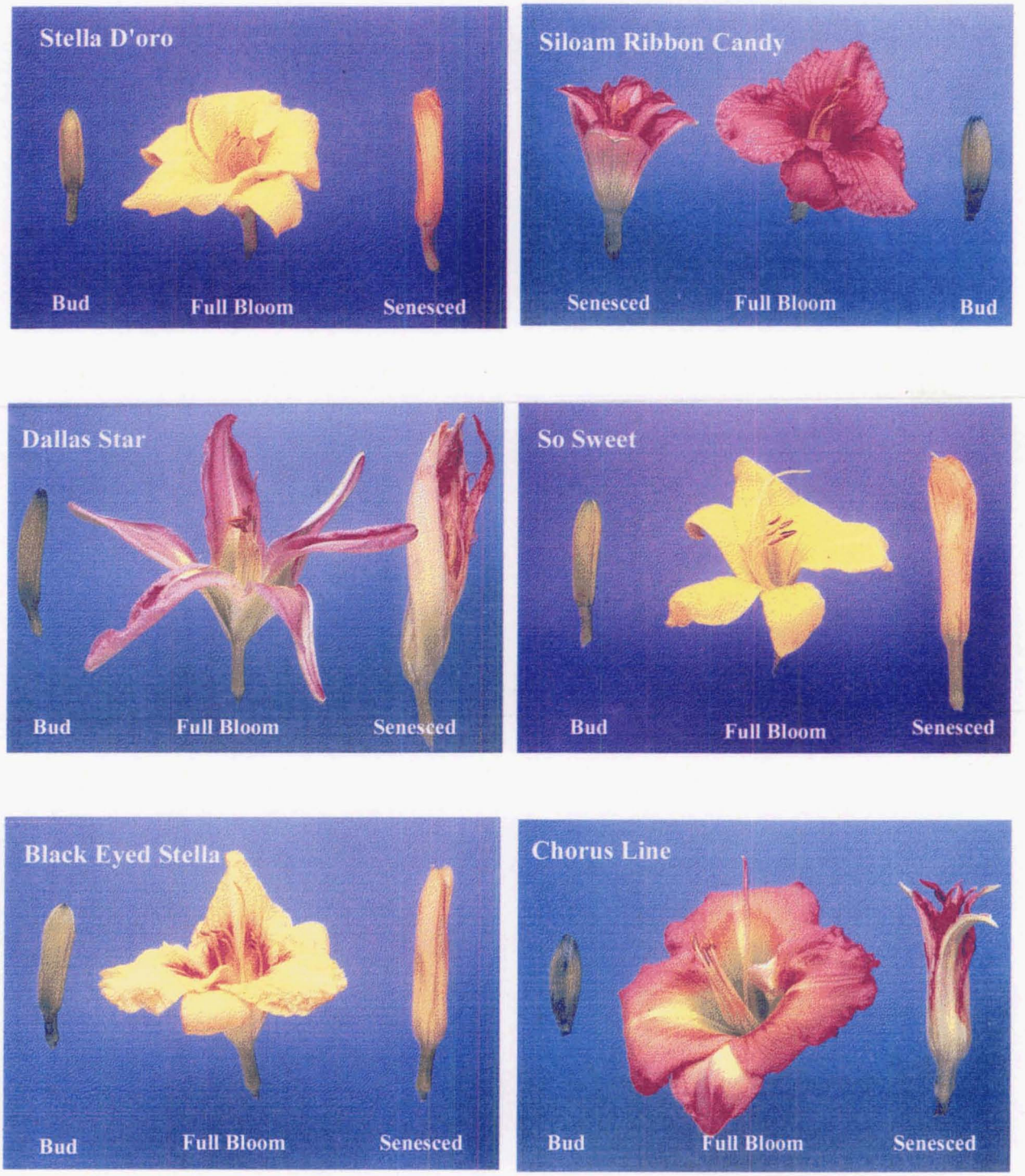
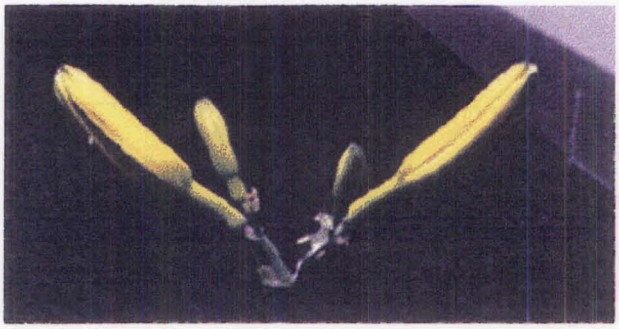
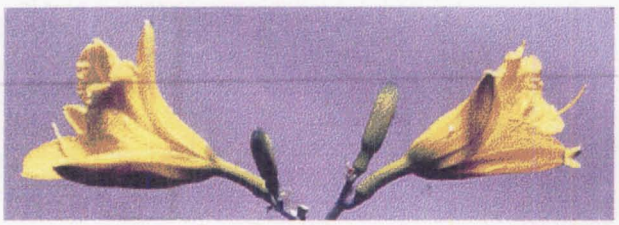


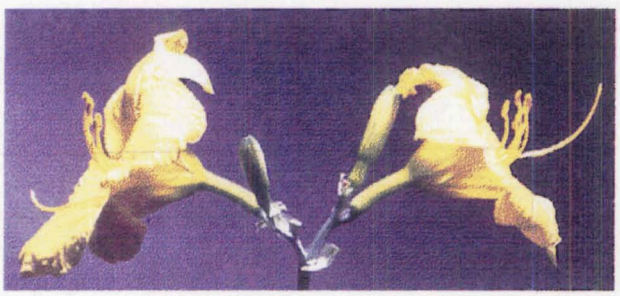
Plate 3.2
Major developmental stages of daylily flowers of the cultivars Stella D'oro, Siloam Ribbon Candy, Dalls Star, So Sweet, Black Eyed Stella and Chorus Line. Bud: buds collected 2 days before opening; Full Bloom: fully open flowers collected on the day of opening; Senesced: senesced flowers collected one day after their opening.



1: Bud (-24 h)

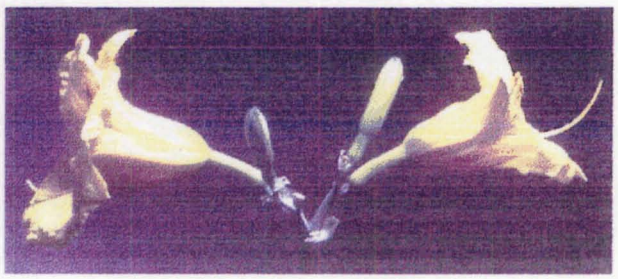


2: Bud (-12 h)

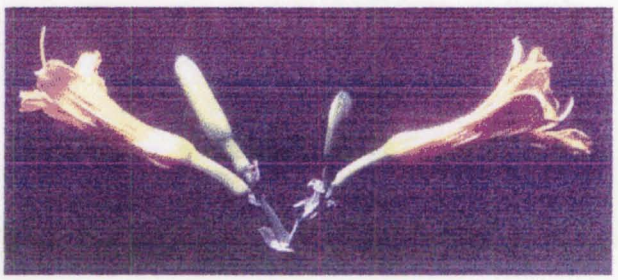


3: Full Bloom (0 h)

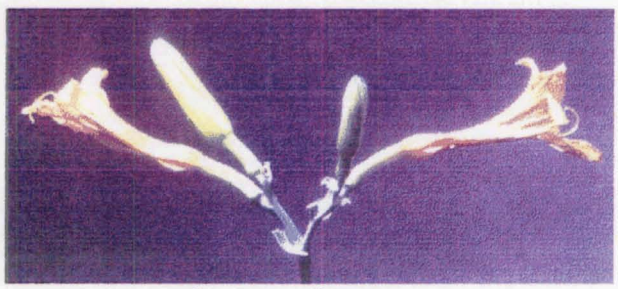
4: Full Bloom (6 h)



5: Full Bloom (12 h)



6: Senesced (24 h)



7: Senesced (48 h)

Plate 3.3
A closer examination of the developmental stages of daylily cv.Stella D'oro flowers borne on the glasshouse-grown plants. Bud: buds observed on the day before opening; Full Bloom: fully open flowers observed on the day of opening; Senesced: wilted flowers observed 1 and 2 days after opening. Time 0 h represents flowers at full bloom stage observed at 9 a.m. on the day of opening. Numbers 1 to 7 indicate the different stages of development.

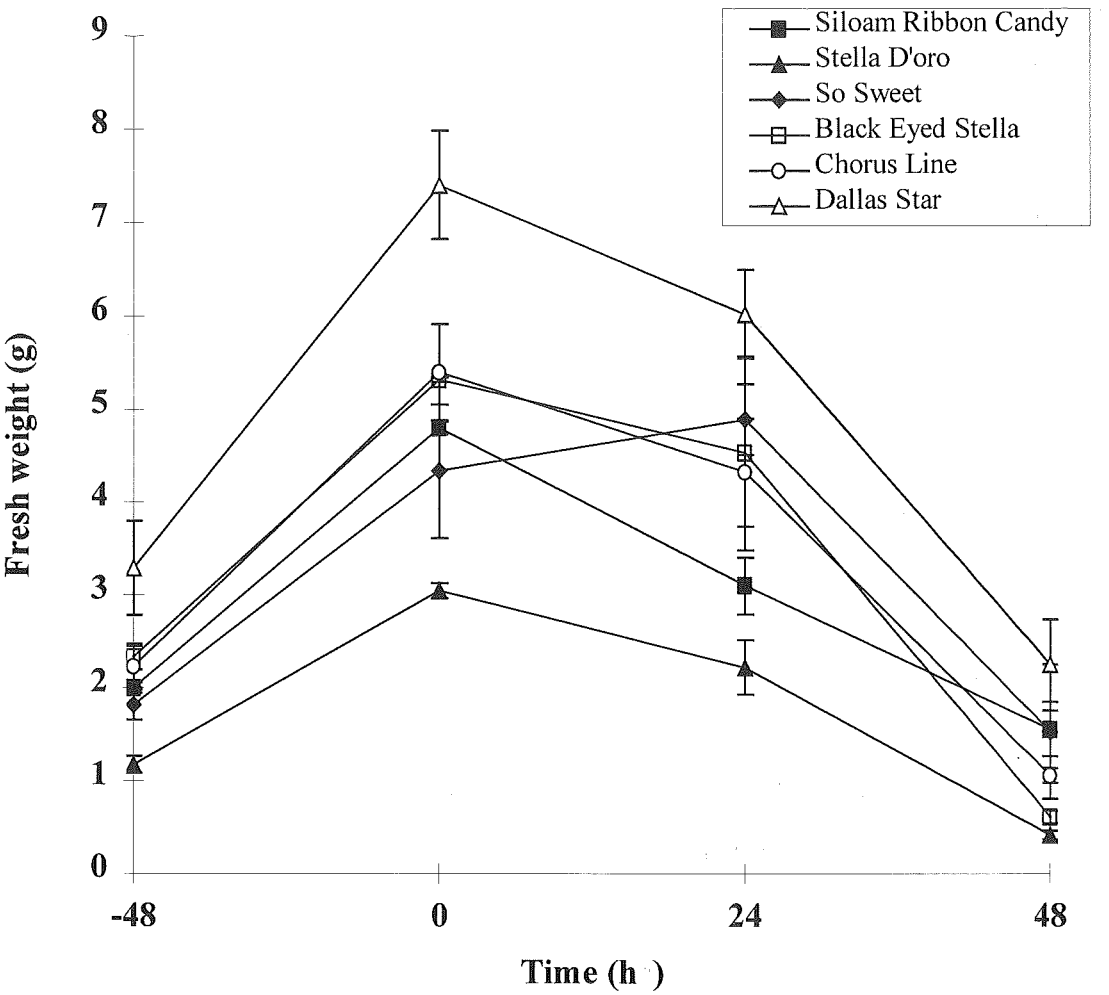


Figure 3.1: Change in fresh weight during development and senescence of daylily flowers. Time –48 h represents buds collected 2 days before flower opening while time 0 h represents flowers at full bloom stage collected at 9 a.m. on the day of flower opening. Time 24 h and 48 h represent wilted flowers collected at 9 a.m., 1 and 2 days after the full bloom stage respectively. Error bars represent standard deviation for three flowers.

Table 3.9: Soluble protein content in petals of cvs. Stella D'oro, Chorus Line and Dallas Star at three developmental stages of flowers.

	Soluble protein content (mg/flower)		
Stage of the flower	**Stella D'oro**	**Chorus Line**	**Dallas Star**
Bud (-48 h)	28.6 ± 9.7	13.7 ± 1.4	21.8 ± 7.2
Full Bloom (0 h)	60.1 ± 8.9	16.7 ± 1.8	21.4 ± 2.3
'Senesced' (24 h)	2.4 ± 3.6	8.5 ± 1.3	14.4 ± 3.2

Proteins from buds and flowers were extracted in phosphate buffer (0.01M) at pH 7. Errors represent standard deviation for three flower extracts. Three replicate determinations were carried out with each extract.

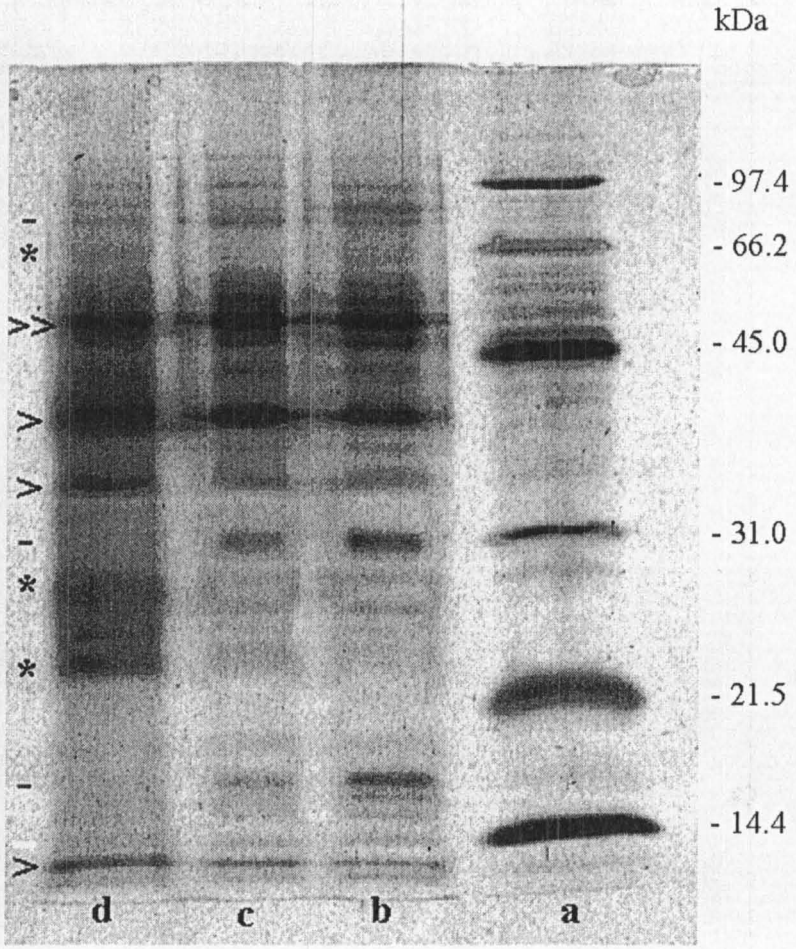


Plate 3.4
Change in soluble proteins during development of flowers of cv. Stella D'oro. After SDS-PAGE, the gels were subjected to silver staining. Proteins were extracted in phosphate buffer (0.01 M) at pH 7. Lanes b, c and d had been loaded with 11 mg of tissue fresh weight -: bands dissapear during senescence; *: bands appear during senescence; >: bands increase in strength during senescence; >>bands do not seem to change during senescence. Lane a: molecular weight markers; lane b: bud collected on the day before opening; lane c: fully open flower collected on the day of opening; lane d: Senesced flower collected on the day after opening.

3.3 Effect of Different Chemicals on the Senescence of cv. Stella D'oro

3.3.1 Effect of Chemicals on the Senescence of Buds, Flowers and Petals

3.3.1.1 Effect of Cycloheximide, Ethrel, Silver Thiosulfate, Ascorbic acid, Casein Hydrolysate, Glyphosate and 8-hydroxyquinoline

Cut full bloom flowers of cv. Stella D'oro always wilted 24 h after the pedicel base was immersed in water (Plate 3.5A). Floral buds detached two days before opening did not open in water. Furthermore, even the buds harvested on the day before full bloom did not always open fully when treated with water. The term 'open' has been used in this section to describe the unfolding of petals from buds. Sometimes they opened fully after 24 h in water but in other instances they opened only ¼ or ½ (Plate 3.5B). Therefore, buds were not very good materials to study the effect of chemicals on flower longevity, although they can be used to confirm the response of fully open flowers. On the other hand, detached petals from buds collected on the day before opening, unfolded along the two sides, turned yellow and enlarged to about 4 times the original size after 24 h of incubation in water (Plate 3.6A). Inner petals from fully open flowers treated with water for 24 h became watery, transparent and shrunk (Plate 3.6B), while outer petals from the same became slightly dry, transparent and shrunk.

Cycloheximide, an inhibitor of protein synthesis, inhibited bud opening and flower senescence (see Plate 3.5 and Table 3.10A). This effect was also seen in detached petals collected from buds and full bloom flowers (see Plate 3.6 and Table 3.10B). The detached petals from open flowers remained fresh and firm, while those from buds remained closed, and did not enlarge after 24 h of this treatment. In addition, the effect of cycloheximide on flowers and buds were dose dependent: higher concentrations resulted in less senescence of fully open flowers and less opening of buds than the lower concentrations.

Full bloom flowers treated with ethrel (see Table 3.10A) showed similar symptoms to those treated with water. Similar to cycloheximide, ethrel also appeared to inhibit bud opening. This effect was confirmed by the observation that petals detached from buds when incubated in an ethrel solution also failed to open and enlarge (see Table 3.10B).

The changes of fully open flowers, buds and detached petals treated with silver thiosulfate, ascorbic acid, casein hydrolysate, glyphosate and 8-hydroxyquinoline were not different from those treated with water.

3.3.1.2 Effect of Sugars

Sucrose, fructose and glucose neither delayed nor enhanced senescence of fully open flowers (Table 3.11) or detached petals from flowers. Sugar-treated buds and petals from buds also did not show a difference to those from water controls except resulting in slightly less expanded petals under certain conditions. Sizes of the inner petals from fructose (both 300 and 800 mg/L) and glucose (800 mg/L) and, outer petals from sucrose (300 and 800 mg/L), fructose (800 mg/L) and glucose (800 mg/L) were between ½ and ¾ the size of water control.

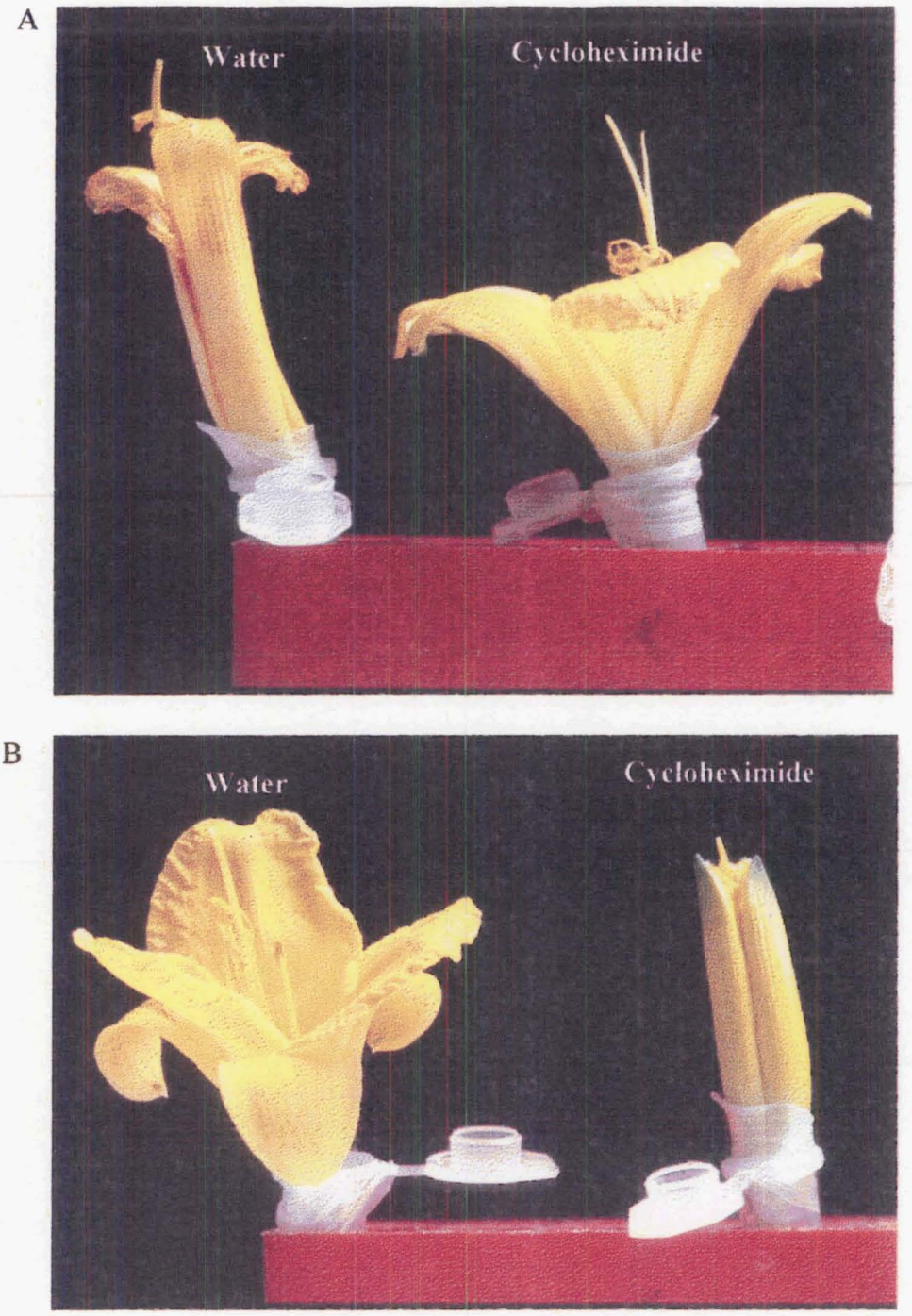


Plate 3.5
Bud opening and longevity of detached flowers of cv. Stella D'oro, 24 h after the treatment with water or 1 mg/mL cycloheximide. A: full bloom flowers; B: buds. Full bloom flowers were collected on the day of opening while buds were collected on the day before opening.

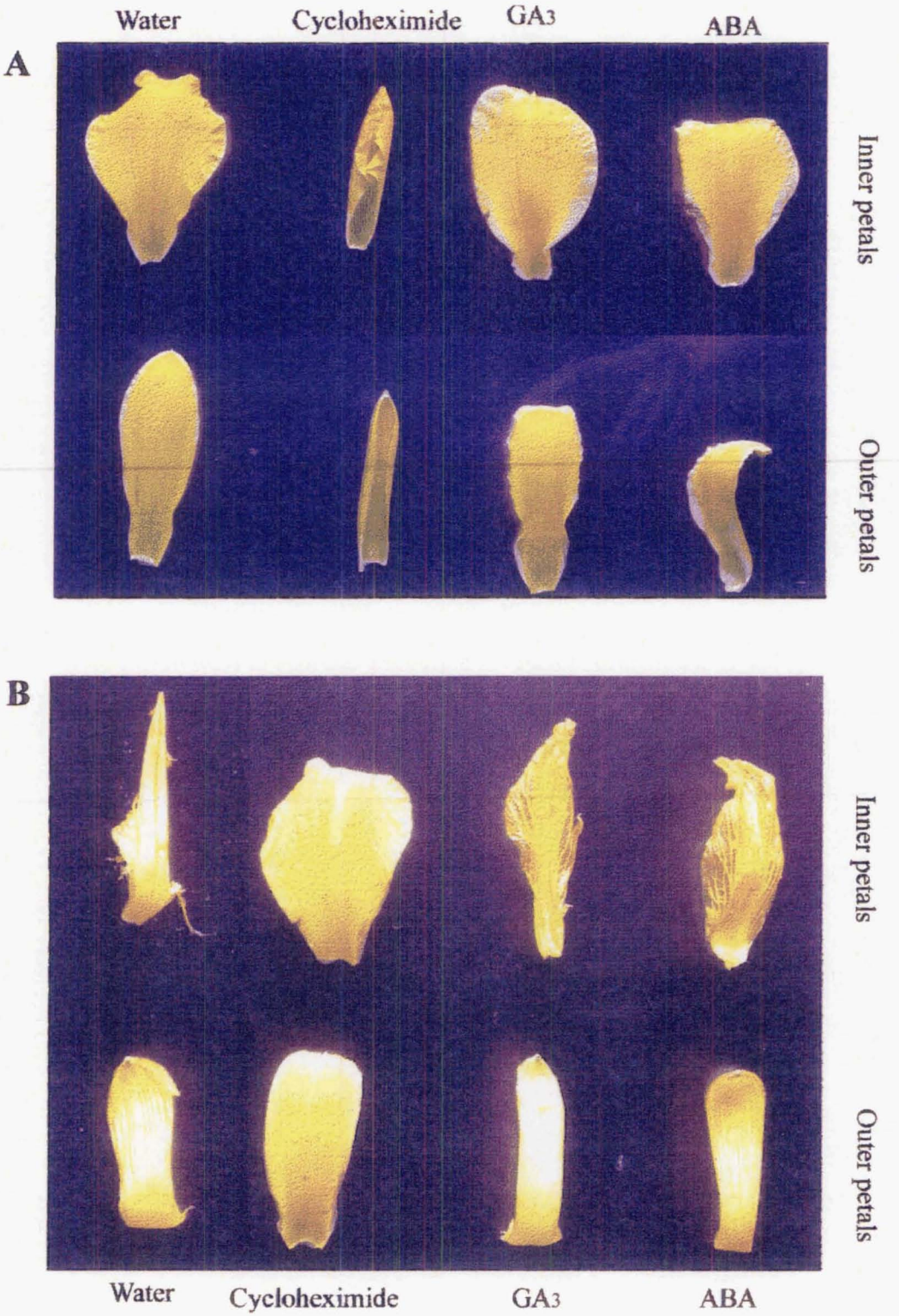


Plate 3.6
Effect of different chemicals (1 mg/mL) on isolated petals of cv. Stella D'oro. A: buds (collected on the day before opening); B: full bloom flowers (collected on the day of opening). Photos were taken 24 h after the start of the chemical treatment.

Table 3.10A: Effect of different chemicals on buds and full bloom flowers of cv. Stella D'oro.

Compound	Concentration (mg/L)	Observation	
		Full bloom flowers	**Buds (-24 hr)***
Water	-	Completely wilted.	¼ -Fully open.
Cycloheximide	0.01	Fully open. Upper 3/4 of inner petals and margins of outer petals transparent.	Slightly open (1/8) at the tip.
Cycloheximide	0.1	Fully open. Upper 1/2 of inner petals and margins of outer petals transparent.	Slightly open (1/16) at the tip.
Cycloheximide	1000	Fully open. Margins of the inner petals were slightly transparent. Senescence symptoms appeared gradually. Flowers lasted 2 days before being fully wilted.	Closed.
Ethrel	14.5 & 72.3	Completely wilted.	n.t.
Ethrel	144.5	Completely wilted but 1/8-1/4 open.	1/8 open.
Silver thiosulfate	125 & 250	Completely wilted.	Fully open.
Silver thiosulfate	5000	Completely wilted.	Full-3/4 open.
Ascorbic acid	250	Completely wilted.	½-1/4 open.
Ascorbic acid	500	Completely wilted.	Full-3/4 open.
Ascorbic acid	1000	Completely wilted.	¾-1/8 open.
Casein hydrolysate	250	Completely wilted.	¼-3/4 open.
Casein hydrolysate	500	Completely wilted.	¾ open.
Glyphosate	360	Completely wilted.	1/8-1/2 open.
Glyphosate	728, 1800 & 18,000	Completely wilted.	n.t.
8-hydroxyquinoline	200	Completely wilted.	¼-fully open.

Buds were slightly open at the tip at the time of collection from the plant. After the cut ends of the pedicels of buds or flowers were immersed in the appropriate solutions for 24 h, observations were taken from 3 replicates. n.t.: not tested. *: The response is given as a range when the 3 replicate buds behaved differently.

Table 3.10B: Effect of incubating isolated petals from buds and full bloom flowers of cv. Stella D'oro in different chemical solutions.

Compound	Concentration (mg/L)	Observation			
		Full bloom flowers		Buds	
		Inner petals	Outer petals	Inner petals	Outer petals
Water or other chemicals#	-	Watery, transparent, shrunk.	Slightly dry, transparent, shrunk.	Open and enlarged; yellow.	Open and enlarged; yellow.
Cycloheximide	1	Fresh.	Fresh.	Closed; not enlarged.	Closed; not enlarged.
Ethrel	144.5	Watery, transparent, shrunk.	Slightly dry, transparent, shrunk.	Closed, not enlarged.	Closed, not enlarged.
Casein hydrolysate	250	Watery, transparent, shrunk.	Slightly dry, transparent, shrunk.	¾ open; 1/2 the size of water control; Yellow.	½ open; 1/2 the size of water control; yellow.

After treatment for 24 h, observations were taken from 2-3 replicates. #:silver thiosulfate, ascorbic acid, casein hydrolysate, glyphosate and 8-hydroxyquinoline tested at the concentrations mentioned in the table 3.10A.

Table 3.11: Effect of sugars on buds and full bloom flowers of cv. Stella D'oro.

Compound	Concentration (mg/L)	Observation	
		Full bloom flowers	**Buds (-24 h)***
Water	-	Completely wilted.	3/4 open.
Sucrose	300	Completely wilted.	½-1/4 open.
Sucrose	800	Completely wilted.	¼-1/8 open.
Sucrose	100 pulsed for 10 & 60 min and transferred to water.	Completely wilted.	n.t.
Fructose	300	Completely wilted.	1/8 open.
Fructose	800	Completely wilted.	¼ open.
Glucose	300	Completely wilted.	¼-1/8 open.
Glucose	800	Completely wilted.	1/2 open.

Buds were slightly open at the tip at the time of collection from the plant. Buds and flowers were kept in the chemical for 24 h before taking the observation. Observations were taken from 3 replicates. n.t.: not tested. *: The response is given as a range when the 3 replicate buds behaved differently.

3.3.1.3 Effect of Growth Regulators

Growth regulators IAA, BA, GA_3, ABA and kinetin at concentrations tested did not produce any symptoms different to water controls (see Plate 3.6 and Table 3.12) when tested with buds, full bloom flowers and detached petals. At first, the growth regulators were found to have extremely high or low pH, which caused the blackening of the flower parts submerged in growth regulators and bending of the tube. Therefore, in later experiments the pHs of the solutions were adjusted to about pH 6.0. Flowers, buds and petals kept in the above mentioned growth regulators with adjusted pH also did not show any difference to those kept in water controls.

3.3.1.4 Effect of Metal Ions

Since metals ions, specially the Mn^{++} appeared to enhance the LAP activity in petal extracts (see section 3.4.8.2), and LAP appeared to have a role in flower senescence (see section 3.4.3), the effect of metal ions on flower longevity was tested using $MnSO_4$ (1 and 10 mM), $ZnSO_4$ (10 mM) and $MgSO_4$ (10 mM). However, these metal ions at the concentrations tested did not have any effect on fully open flowers or petals from fully open flowers when compared to water controls, i.e. the time course of senescence was the same in all these treatments.

Table 3.12: Effect of growth regulators on buds and full bloom flowers of cv. Stella D'oro.

Compound	Concentration (mg/L)	Observation	
		Full bloom flowers	**Buds (-24 h)***
Water	-	Completely wilted.	3/4 open.
IAA	1 & 5	Completely wilted.	n.t.
BA	25	Completely wilted.	¼ open.
BA	50	Completely wilted.	1/6-1/2 open.
GA_3	1 & 5	Completely wilted.	n.t.
GA_3	3.5	Completely wilted.	¼-1/2 open.
GA_3	35	Completely wilted.	1/2 open.
Kinetin	25	Completely wilted.	¾-fully open.
Kinetin	50	Completely wilted.	¼-1/2 open.
ABA	22.5	Completely wilted.	n.t.
ABA	225	Completely wilted.	¼-fully open.

Buds were slightly open at the tip at the time of collection from the plant. After the cut ends of the pedicels of buds and flowers were immersed in the approriate solutions for 24 h, observations were taken from 3 replicates. n.t.: not tested. *: The response is given as a range when the 3 replicate buds behaved differently.

3.3.2 Effect of Chemicals on the Senescence of Leaves

3.3.2.1 Effect of Cycloheximide, Ethrel, Silver Thiosulfate, Ascorbic acid, Casein Hydrolysate, Glyphosate and 8-hydroxyquinoline

Leaf discs from cv. Stella D'oro were treated with various chemicals to observe their effect on leaf senescence. Yellowing of leaves was assumed to be the visible symptom of senescence while browning was assumed to be due to toxic effect of the chemical. Browning of leaf discs was observed with high concentrations of certain chemicals (cycloheximide, ethrel, silver thiosulfate, glyphosate and 8-hydroxyquinoline); hence lower concentrations were preferred. Even after 14 days of treatment, there was no smell or decaying of leaves indicating absence of microbial contamination. The appearance of leaf discs treated with different chemicals for 14 days are shown in Plate 3.7. Leaf discs kept in water began to yellow at around 8^{th} day. Cycloheximide at 1000 mg/L caused browning of the tissue, presumably this concentration being toxic. At concentrations 0.01 and 0.1 mg/L, leaf discs remained green even after 14 days in cycloheximide (Table 3.13). Ethrel caused browning of leaf discs at concentrations between 72.5 to 144.5 mg/L. At lower concentrations between 0.14 to 14 mg/L, leaf discs remained green even after 14 days. However, the leaf discs in the ethrel solutions turned flaccid, when compared to those in the following treatments: fresh, untreated leaf discs (turgid), those treated with cycloheximide, silver thiosulfate and water. Leaf discs treated with silver thiosulfate, ascorbic acid, casein hydrolysate and glyphosate started to yellow at around the same time as those kept in water. However, it is worth noting that the high concentration of casein hydrolysate tested (i.e. 500 mg/L) appeared to reduce the yellowing of leaf discs after 14 days. Leaf discs kept in 200 mg/L 8-hydroxyquinoline exhibited browning. Even at 100 mg/L it exhibited some browning of discs on 6.3 ± 1.4 days.

Plate 3.7:
Effect of different chemicals on leaf discs of cv. Stella D'oro 14 days after incubating with the chemical. A: water (control); B: ethrel (0.14 mg/L); C: ethrel (1.4 mg/L); D: STS (0.25 mg/L); E: STS (2.5 mg/L); F: STS (25 mg/L); G: GA_3 (25 mg/L); H: GA_3 (50 mg/L); I: BA (25 mg/L); J: BA (50 mg/L); K: cycloheximide (0.1 mg/L).

Table 3.13: Effect of different chemicals on leaf discs of cv. Stella D'oro.

Compound	Concentration (mg/L)	Appearance of 1st yellowing symptom (days)#	Appearance after 14 days
Water	-	8.4 ± 0.75	Yellow (80-100%)
Cycloheximide	0.01 & 0.1	not detected. #6	Green (100%)
Ethrel	0.14, 1.4 & 14	not detected. #6	Green (100%)
Silver thiosulfate	0.25	8.3 ± 2.4	Yellow (100%)
Silver thiosulfate	2.5	7.0 ± 3.0	Yellow (75%)
Silver thiosulfate	25	7.3 ± 2.9	Yellow (75%)
Ascorbic acid	250	8.8 ± 2.6	Yellow (90%)
Ascorbic acid	500	10.2 ± 1.7	Yellow (90%)
Ascorbic acid	1000	11.5 ± 3.0	Yellow (50%)
Casein hydrolysate	250	8.0 ± 1.8	Yellow (75%)
Casein hydrolysate	500	9.5 ± 1.8	Yellow (50%)
Glyphosate	360	9.8 ± 1.2	Yellow (75%)
8-hydroxyquinoline	100	6.3 ± 1.4 (brownish)	Brownish Yellow (75%)

The values indicate averages of 6 replicates, each with 2 discs per replicate together with their respective standard deviations. The experiment was repeated twice. The leaf discs were inspected daily for the appearance of yellowing. #: The number of the leaf discs remaining green after 14 days of incubation in the chemical.

3.3.2.2 Effect of Sugars

Leaf discs treated with both sucrose and fructose at the concentrations tested remained green much longer than those kept in water (Table 3.14). At the end of 14 days all the discs were almost green except the discs in 300 mg/L fructose which showed some yellowing. On the other hand, leaf discs incubated in glucose turned brownish around the same time as those incubated in water containing 100 mg/L 8-hydroxyquinoline (to minimize bacterial growth during long term incubation). This browning appears more likely to be due to the effect of 8-hydroxyquinoline rather than the effect of glucose.

3.3.2.3 Effect of Growth Regulators

Of the growth regulators tested, ABA caused yellowing of leaf discs considerably earlier when compared to discs incubated in water (Table 3.15). Discs treated with BA remained green until the end of experimental period. IAA and kinetin caused yellowing of leaf discs around the same time as discs in water, while GA_3 retained the green colour a little longer.

Table 3.14: Effect of sugars on leaf discs of cv. Stella D'oro.

Compound	Concentration (mg/L)	Appearance of 1st yellowing symptom (days)#	Appearance after 14 days
Water	-	8.4 ± 0.75	Yellow (80-100%)
Sucrose	300	not detected. #6	Green (100%)
Sucrose	800	11 ± 0 #5	Green (90%)
Fructose	300	11 ± 0 #2	Yellow (25%)
Fructose	800	not detected. #6	Green (100%)
Glucose in 100 mg/L 8-hydroxyquinoline	300	6.8 ± 2.7 (brownish)	Brownish Yellow (85%)
Glucose in 100 mg/L 8-hydroxyquinoline	800	4.8 ± 1.0 (brownish)	Brownish Yellow (50%)

The values indicate averages of 6 replicates, each with 2 discs per replicate, together with their respective standard deviations. The experiment had been repeated twice. The leaf discs were inspected daily for the appearance of yellowing. #: The number of leaf discs remaining green after 14 days of incubation in the chemical solutions.

Table 3.15: Effect of growth regulators on leaf discs of cv. Stella D'oro.

Compound	Concentration (mg/L)	Appearance of 1st yellowing symptom#	Appearance after 14 days
Water	-	8.4 ± 0.75	Yellow (80-100%)
IAA	0.1	8.3 ± 4.1	Yellow (75%)
IAA	1	9.8 ± 3.6	Yellow (75%)
BA	25	not detected. #6	Green (100%)
BA	50	not detected. #6	Green (100%)
GA_3	25	12.0 ± 1.4	Yellow (50%)
GA_3	50	10.8 ± 1.2	Yellow (50%)
ABA	22.5	3.5 ± 0.8	Yellow (50%)
ABA	225	3.3 ± 0.8	Yellow (75%)
Kinetin	25	10.2 ± 2.9	Yellow (50%)
Kinetin	50	9.2 ± 2.2	Yellow (50%)

The values indicate average of 6 replicates, each with 2 discs per replicate, together with their respective standard deviations. The experiment had been repeated twice. The leaf discs were inspected daily for the appearance of yellowing. #: The number of leaf discs remaining green after 14 days of incubation in the chemical solutions.

3.4 Proteolytic Enzymes

3.4.1 Optimisation of Enzyme Reactions and Effect of pH on Proteolytic Enzymes

For optimisation of proteolytic enzyme assays, a number of aspects were examined including enzyme kinetics with respect to reaction time and volume of the enzyme extract in the reaction mixture, requirement for sulfhydryl protecting compounds in the assay mixture and optimum pH for each enzyme. To determine the need for sulfhydryl groups for enzyme activity, sufhydryl protecting compounds were added either to the extraction buffer or to the enzyme reaction mixture or into both.

Endopeptidase activity in daylily flowers was found to be higher in the presence of sulfhydryl protecting compounds. Presence of 2 mM β-mercaptoethanol in the enzyme reaction mixture approximately doubled the enzyme activity when compared to the activity in the presence of 2 mM β-mercaptoethanol in the extraction buffer. Leaving out 2 mM β-mercaptoethanol altogether from the extraction buffer and the enzyme reaction mixture led to a 5-fold reduction in enzyme activity. In the same way 2 mM cysteine also improved endopeptidase activity. There was a 3-fold increase in activity when cysteine was present in the reaction mixture. Since endopeptidase appeared to need sulfhydryl protecting agents for its activity, 2 mM cysteine had been included in the reaction buffer for endopeptidase in this study. The optimum pH for endopeptidase activity was pH 6 (Figure 3.2).

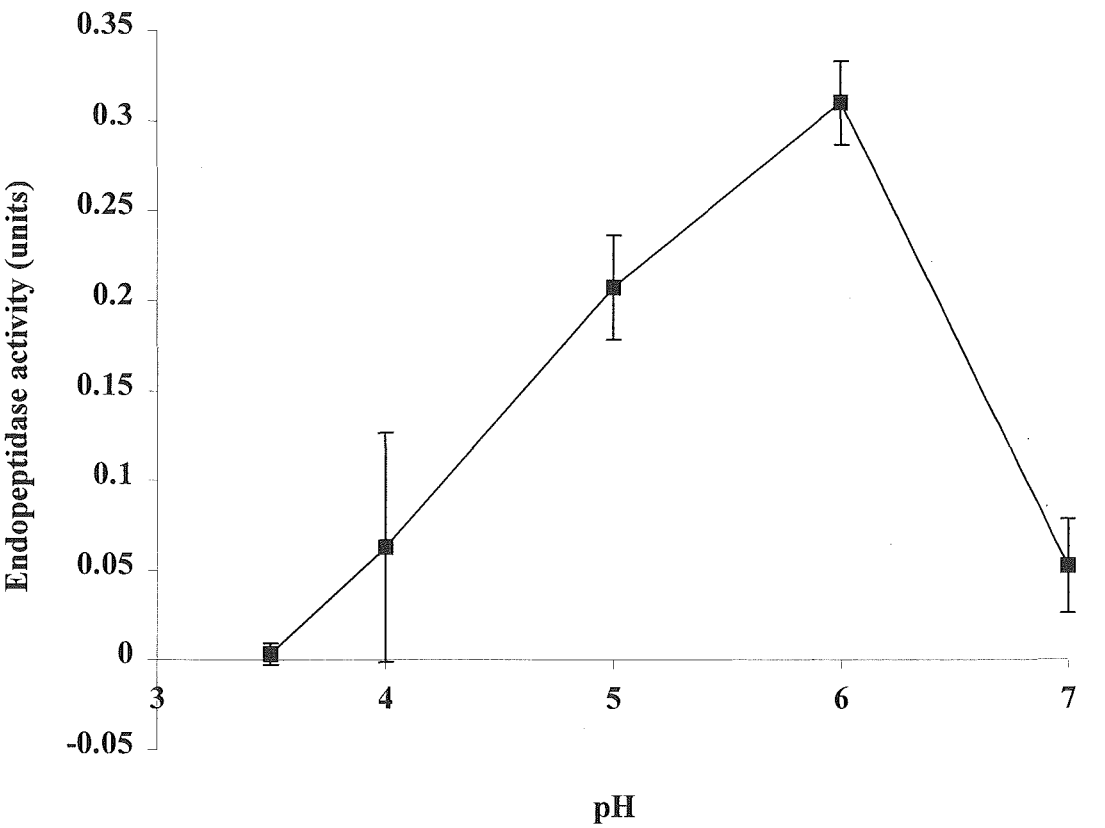


Figure 3.2: Effect of pH on endopeptidase activity in petals of wilted flowers of cv. Stella D'oro collected on the day after opening. One unit of enzyme activity is defined as change in absorbance at 420 nm per h per flower. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with each extract.

Presence or absence of 2 mM β-mercaptoethanol in the extraction buffer or in the enzyme reaction mixture did not affect carboxypeptidase enzyme activity of daylily flowers. When short incubation times (0.5-1 h) and small volumes (10-30 μL) of enzyme extract in the reaction mixture were tested, product formation curves were linear. The optimum pH for the carboxypeptidase was 5 (Figure 3.3).

In the case of leucine aminopeptidase (LAP), presence or absence of 2 mM dithiothretol in the extraction buffer or in the reaction mixture had no effect on the enzyme activity. A linear product formation curve was obtained with 30 min incubation time and 10-30 μL of enzyme extract. The pH curves of the LAP activity in extracts of flowers and leaves showed an optimum at pH 8 (Figures 3.4 and 3.5).

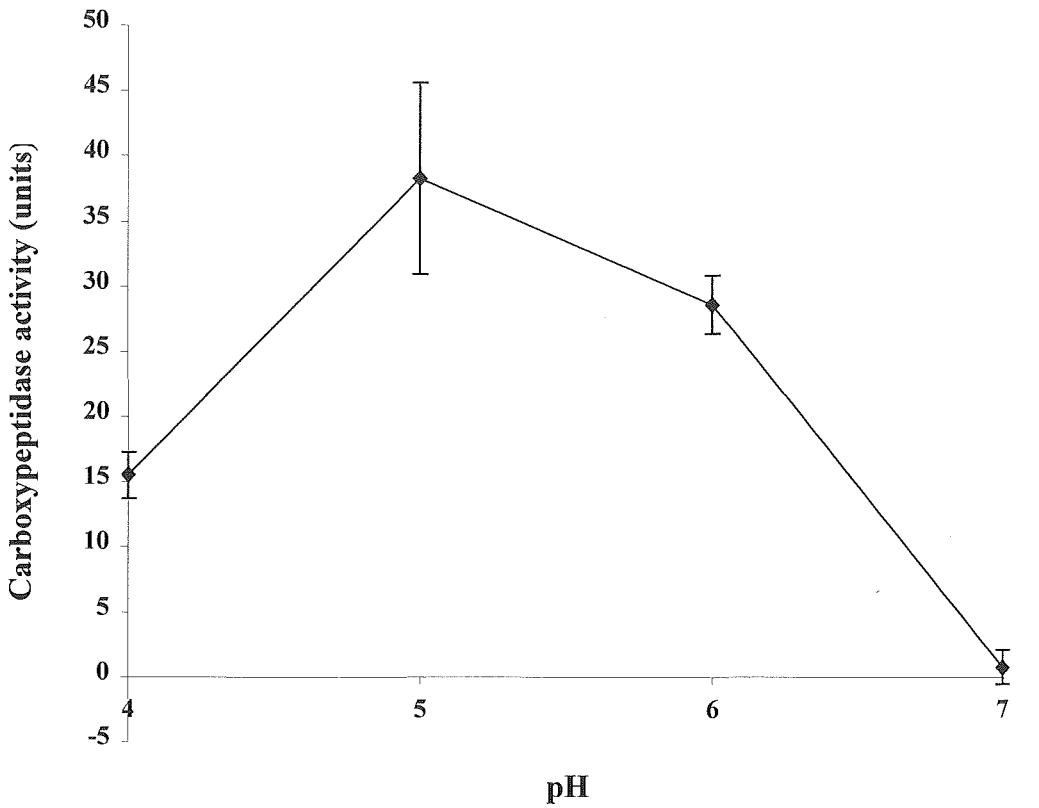


Figure 3.3: Effect of pH on carboxypeptidase activity in petals of full bloom flowers of cv. Stella D'oro. One unit of enzyme activity is defined as change in mg amino acids released per h per flower. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with each extract.

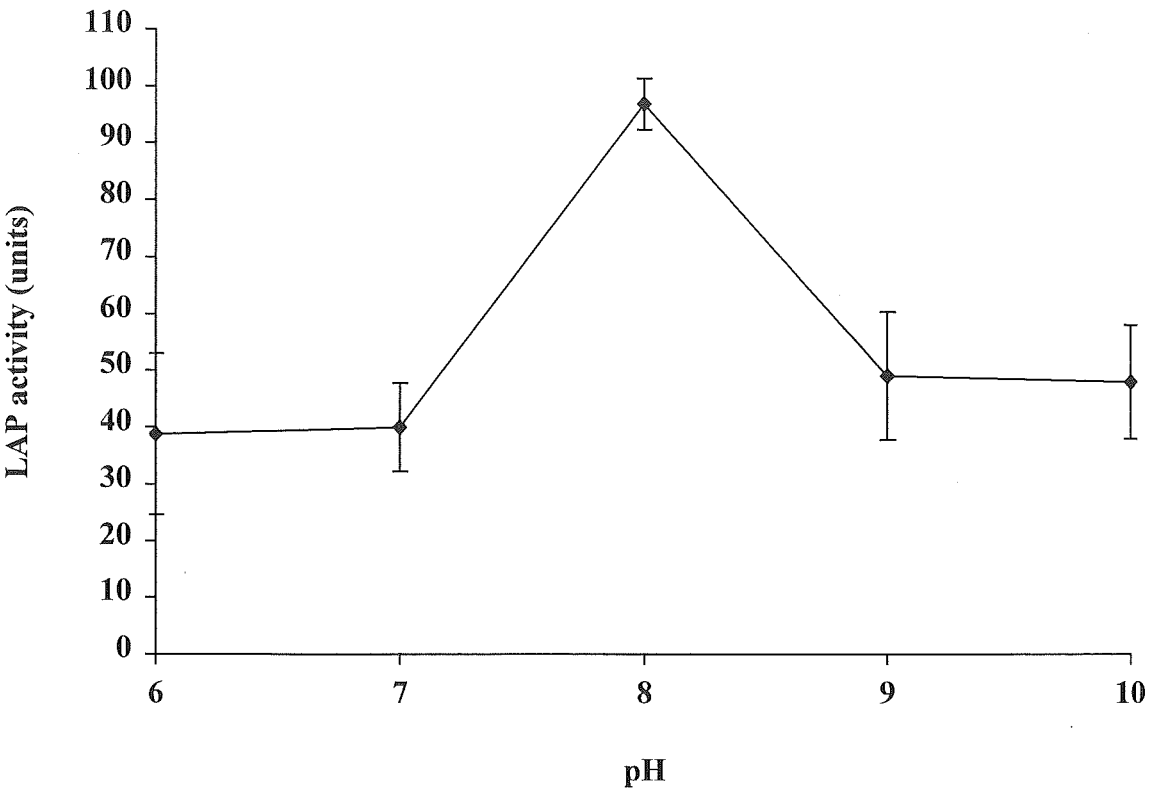


Figure 3.4: Effect of pH on LAP activity in petals of full bloom flowers of cv. Stella D'oro. One unit of enzyme activity is defined as change in absorbance at 410 nm per h per flower. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with each extract.

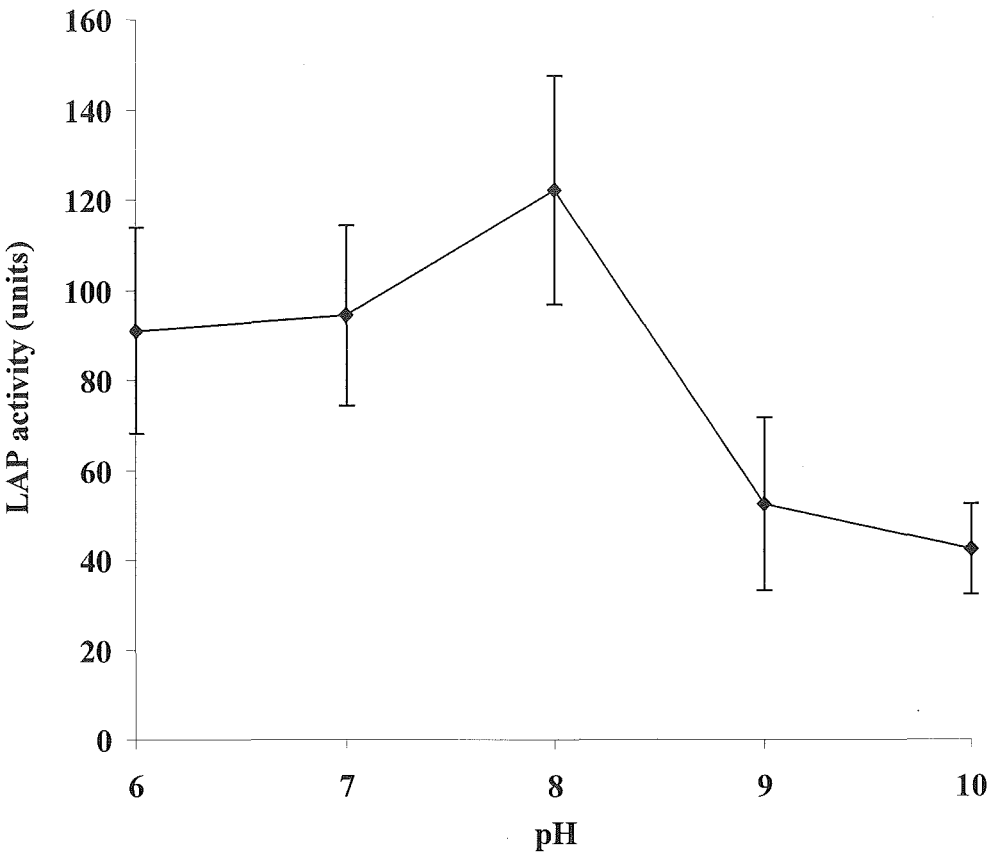


Figure 3.5: Effect of pH on LAP activity in leaves of cv. Stella D'oro. Leaves were collected from 2-3 month old tissue cultured plants. One unit of enzyme activity is defined as change in absorbance at 410 nm per h per g fresh weight. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with each extract.

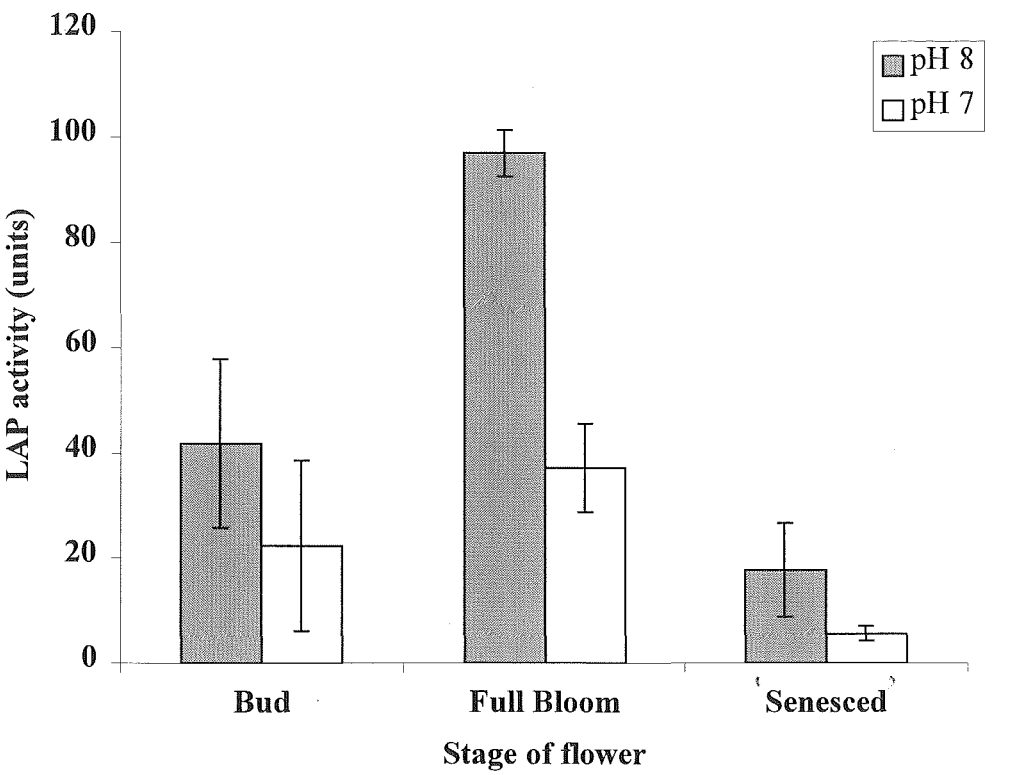


Figure 3.6: Effect of pH 7 and 8 on LAP activity in petals of full bloom flowers of cv. Stella D'oro. Bud: buds collected on the day before opening; Full Bloom: fully open flowers collected on the day of opening; 'Senesced': wilted flowers collected on the day after opening. One unit of enzyme activity is defined as change in absorbance at 410 nm per h per flower. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with each extract.

3.4.2 Effect of Low-Temperature Storage on Proteolytic Activity

For carboxypeptidase and endopeptidase enzymes, effect of storing extracts at low temperatures was determined by freezing the enzyme extracts once at -20°C, and comparing the activity of the extract after thawing out with that of the fresh extract. Carboxypeptidase enzyme lost its activity to undetectable levels after being frozen once. In contrast, activity loss for endopeptidase was only 2.2%.

The effect of low-temperature storage on LAP activity were tested with respect to repeated freezing (and thawing) and storage time as preliminary experiments suggested that LAP in daylily flower extracts was not stable upon freezing. In addition, effect of freezing (-20°C) on LAP isozymes were determined by IEF. The fresh extract lost LAP activity by about 35% (Figure 3.7) when frozen once. After the first freezing (or thawing), the reduction in enzyme activity was lower at subsequent freezing. As much as 54% of the enzyme activity was lost after 4 times of repeated freezing and thawing of the same extracts. This loss in activity was evident in IEF gels too. The two isozyme bands from the frozen extracts were diffused and fainter when compared to those in the fresh extracts (Plate 3.8A and B). IEF on refrigerated extract also produced diffused bands of very low intensity (gel not shown). However, bands produced by fresh enzyme extracts prepared from frozen (-80°C) flowers were similar to those produced by fresh enzyme extracts prepared from freshly detached flowers (gel not shown). This indicates that LAP activity was preserved during -80°C storage of intact flowers. After one day of storage at -20°C, a substantial loss of LAP activity in daylily flower extracts had occurred (Figure 3.8).

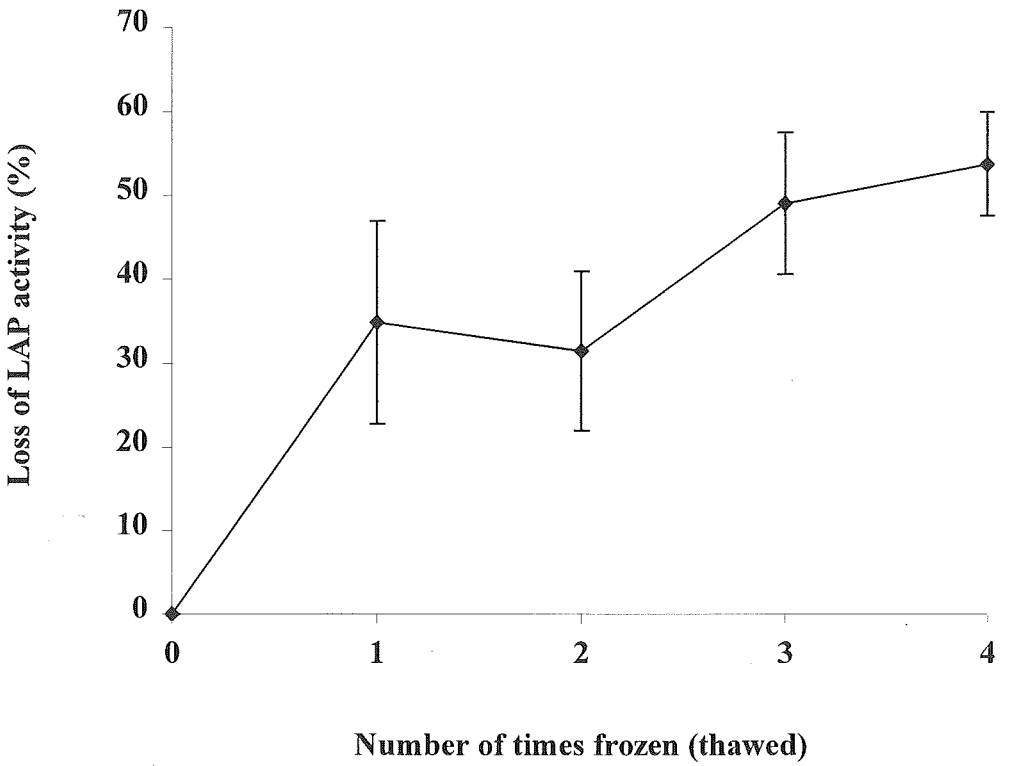
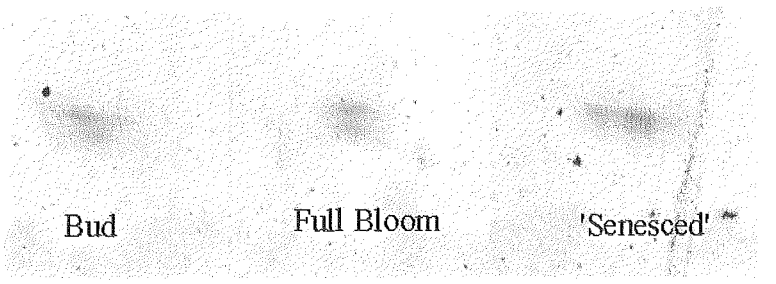


Figure 3.7: Effect of repeated freezing and thawing on LAP activity. Enzyme was extracted from petals of full bloom flowers of cv. Stella D'oro collected on the day of opening. One unit of enzyme activity is defined as change in absorbance at 410 nm per h per flower. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with each extract.

A: Fresh extract



B: Frozen extract

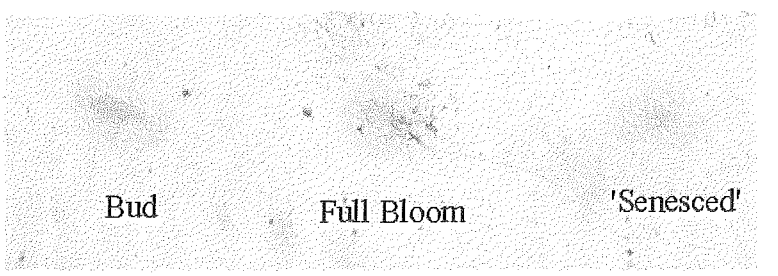


Plate 3.8
IEF gels showing effect of cold storage on LAP isozymes in petals of cv. Stella D'oro. Frozen extracts were stored for 1 day before being subjected to IEF. Bud: bud collected on the day before opening; Full Bloom: fully open flower collected on the day of opening; 'Senesced': wilted flower collected on the day after opening. Each lane in A and B was loaded with 0.03 mg of total proteins.

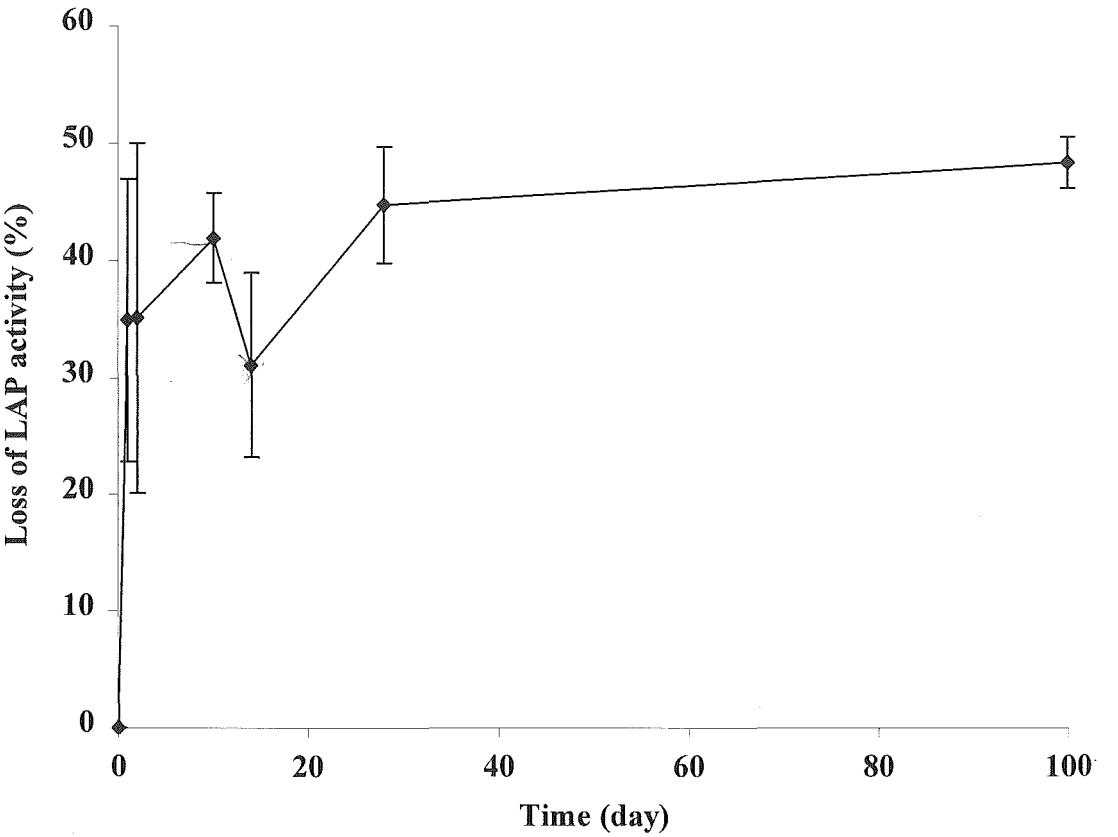


Figure 3.8: Effect of storage time at -20°C on LAP activity. Enzyme extracts were prepared from petals of full bloom flowers of cv. Stella D'oro collected on the day of their opening. One unit of enzyme activity is defined as change in absorbance at 410 nm per h per flower. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with each extract.

3.4.3 Change in Proteolytic Activity during Flower Development

Change in proteolytic enzyme activity during flower development was studied using petals of cv. Stella D'oro flowers collected at different stages of development. The endopeptidase activity in the buds and full bloom flowers collected at 9 a.m. in the morning was very low (Figure 3.9) when azoalbumin was used as the substrate. Afterwards enzyme activity increased dramatically and reached a maximum at 12 h after full bloom. When gelatin was used as the substrate, the enzyme activity was first detected in senesced flowers at 24 h (gel not shown). On the other hand, the LAP activity increased gradually as the flower opened (Figure 3.10) and attained a peak at 6 h after full bloom stage, i.e. 6 h before the endopeptidase peak. In contrast, the activity for the carboxypeptidase remained approximately at the same level throughout flower development and senescence (Figure 3.11).

3.4.4 Units of Enzyme Activity

In an attempt to determine the best way to express changes in enzyme activity during development, the enzyme data had been calculated based on three different parameters, i.e. per flower, per mg soluble protein in the petal extract and per g fresh weight of tissue. The results for endopeptidase, LAP and carboxypeptidase activities are shown in Figures 3.12, 3.13 and 3.14 respectively. The endopeptidase activity was highest at the senesced flower stage, irrespective of the way it was expressed. The highest activity in LAP was found at the full bloom stage when the activity was expressed on the basis of per flower. When the activity was expressed based on the mg soluble protein or g fresh weight, it remained more or less constant at all 3 developmental stages. Carboxypeptidase activity did not change over the three developmental stages studied irrespective of the basis on which it was expressed.

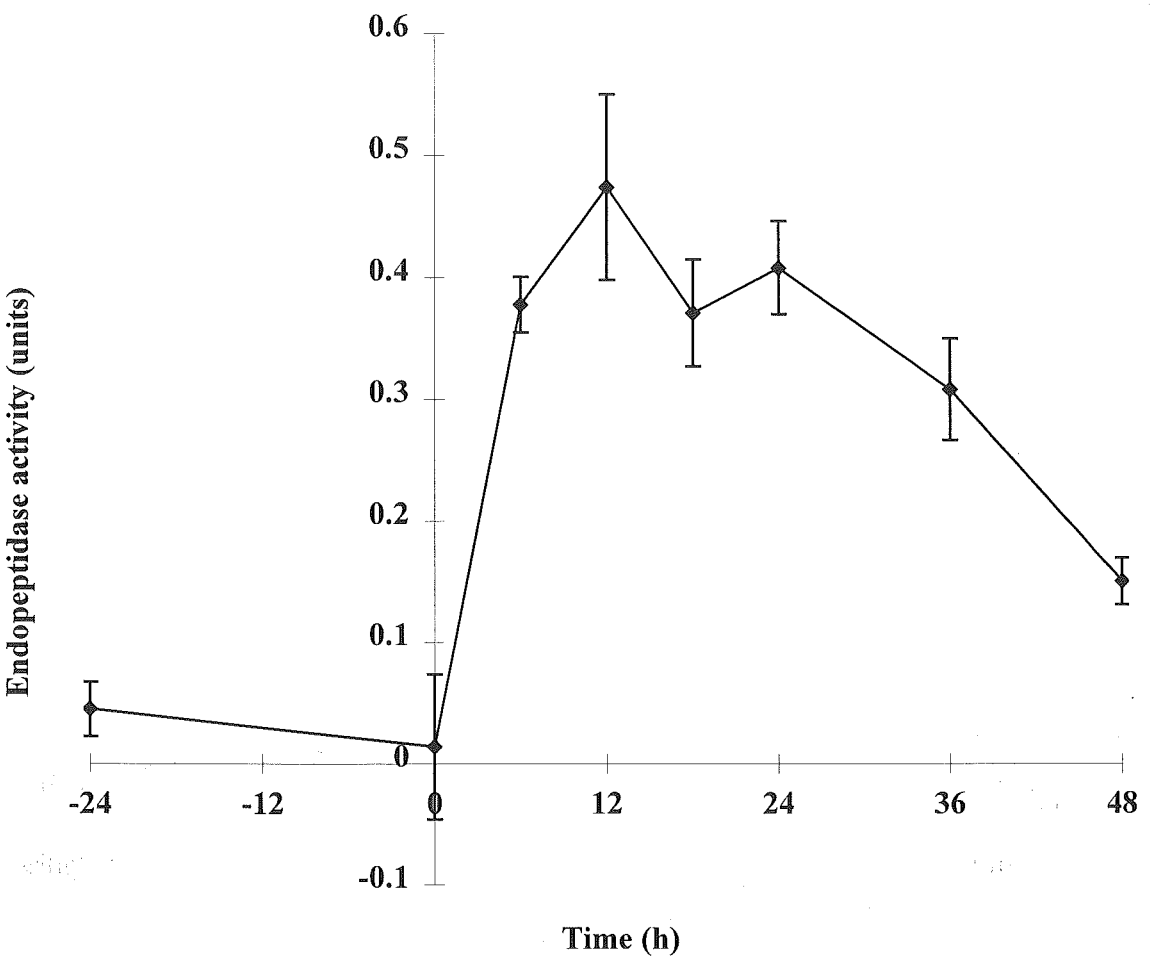


Figure 3.9: Endopeptidase activity in petals of cv. Stella D'oro at different stages of flower development and senescence. One unit of enzyme activity is defined as a change in one unit of change in absorbance at 420 nm per h at 37°C per flower. Time 0 h represents flowers at full bloom stage collected at 9 a.m. on the day of opening. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with each extract.

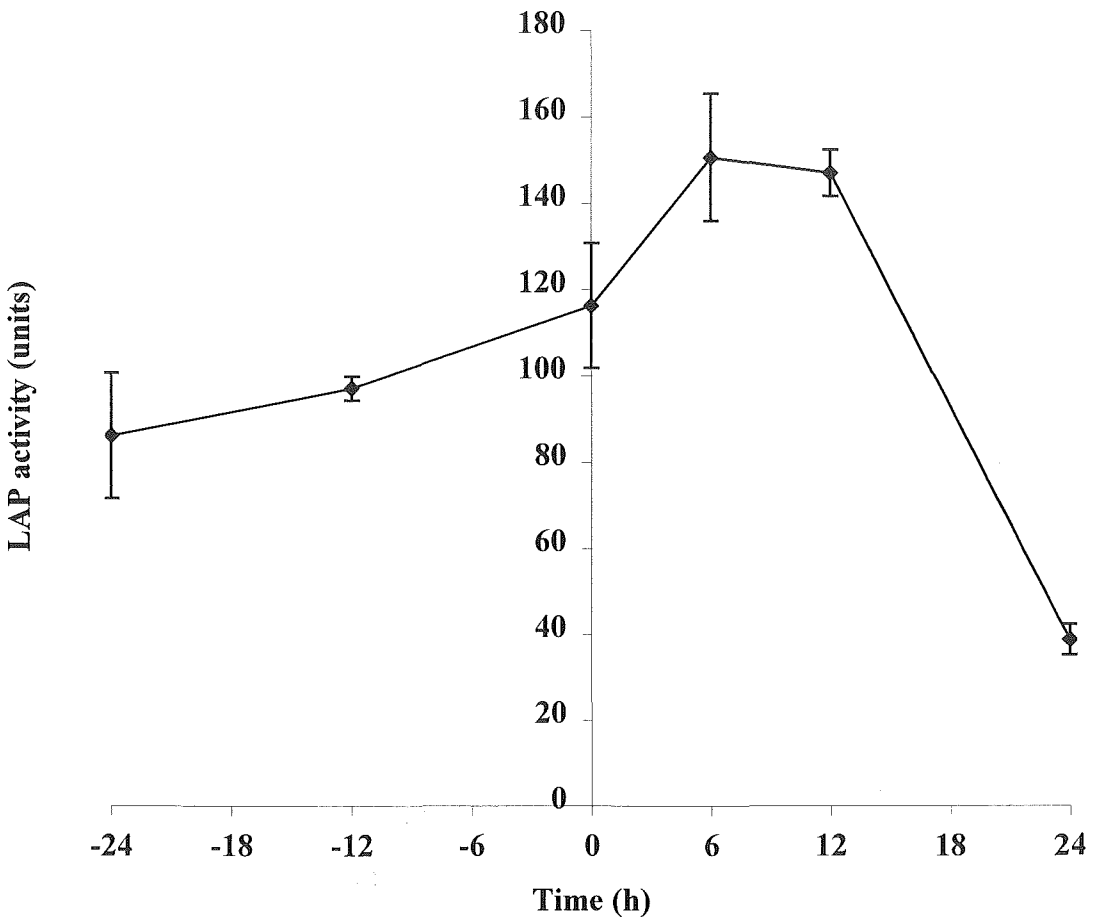


Figure 3.10: LAP activity in petals of cv. Stella D'oro at different stages of flower development and senescence. One unit of enzyme activity is defined as a change in one unit of absorbance at 410 nm per h at 37°C per flower. Time 0 h represents flowers at full bloom stage collected at 9 a.m. on the day of opening. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with each extract.

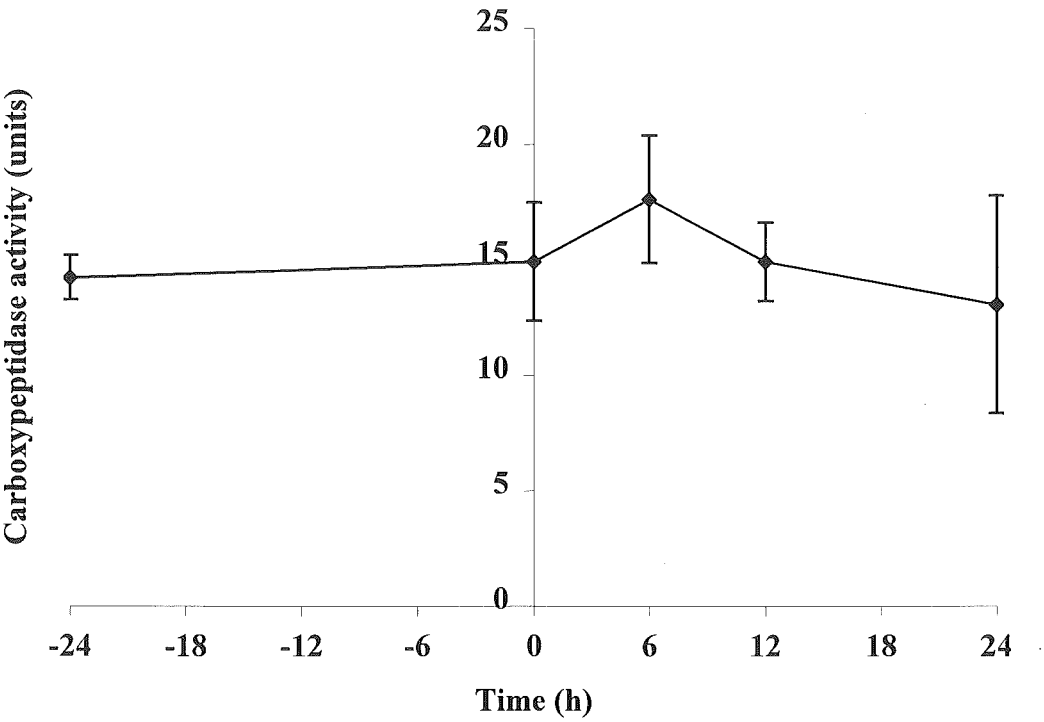


Figure 3.11: Carboxypeptidase activity in petals of cv. Stella D'oro at different stages of flower development and senescence. One unit of enzyme activity is defined as change in mg amino acids produced at 37°C per h per flower. Time 0 h represents flowers at full bloom stage collected at 9 a.m. on the day of opening. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with each extract.

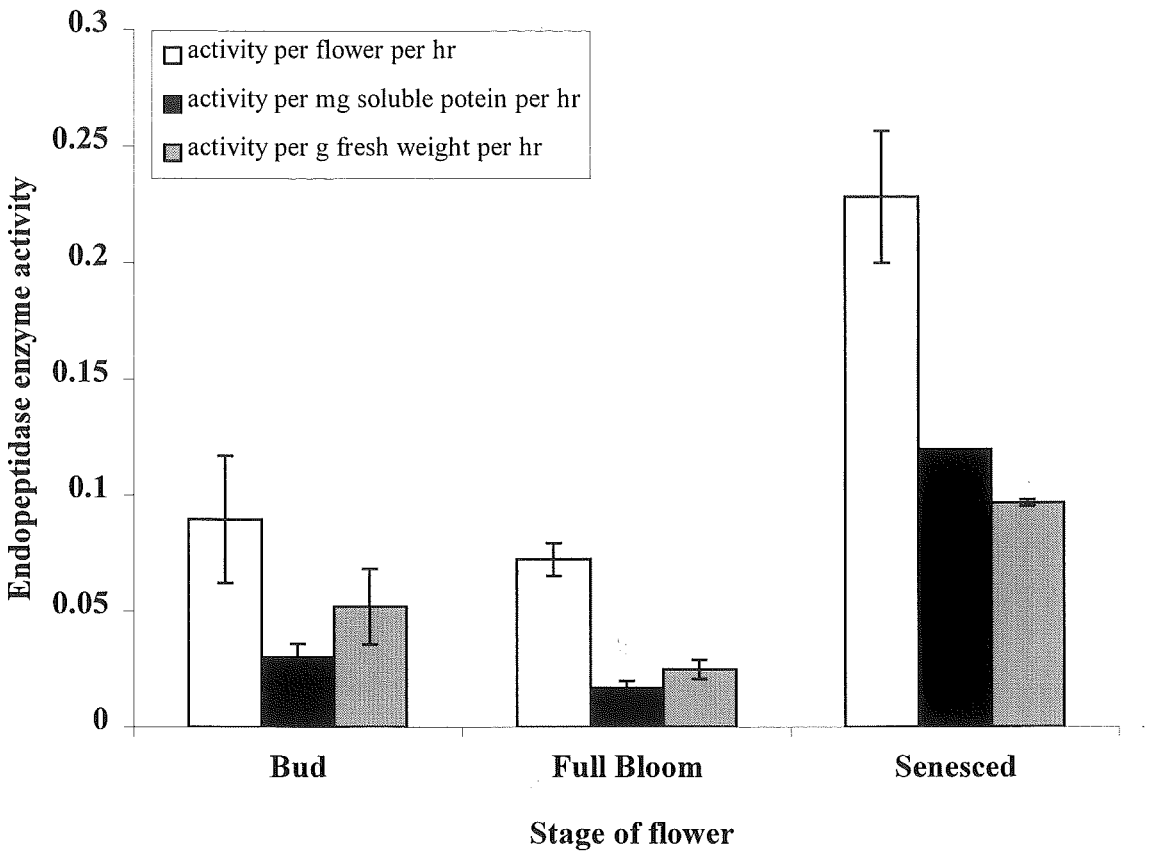


Figure 3.12: Endopeptidase activity in petals of cv. Stella D'oro expressed on the basis of three different parameters. Bud: buds on the day before opening; Full Bloom: fully open flowers collected on the day of opening; 'Senesced': wilted flowers collected on the day after opening. One unit of enzyme activity is defined as a change in one unit of absorbance at 420 nm at 37°C. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with each extract.

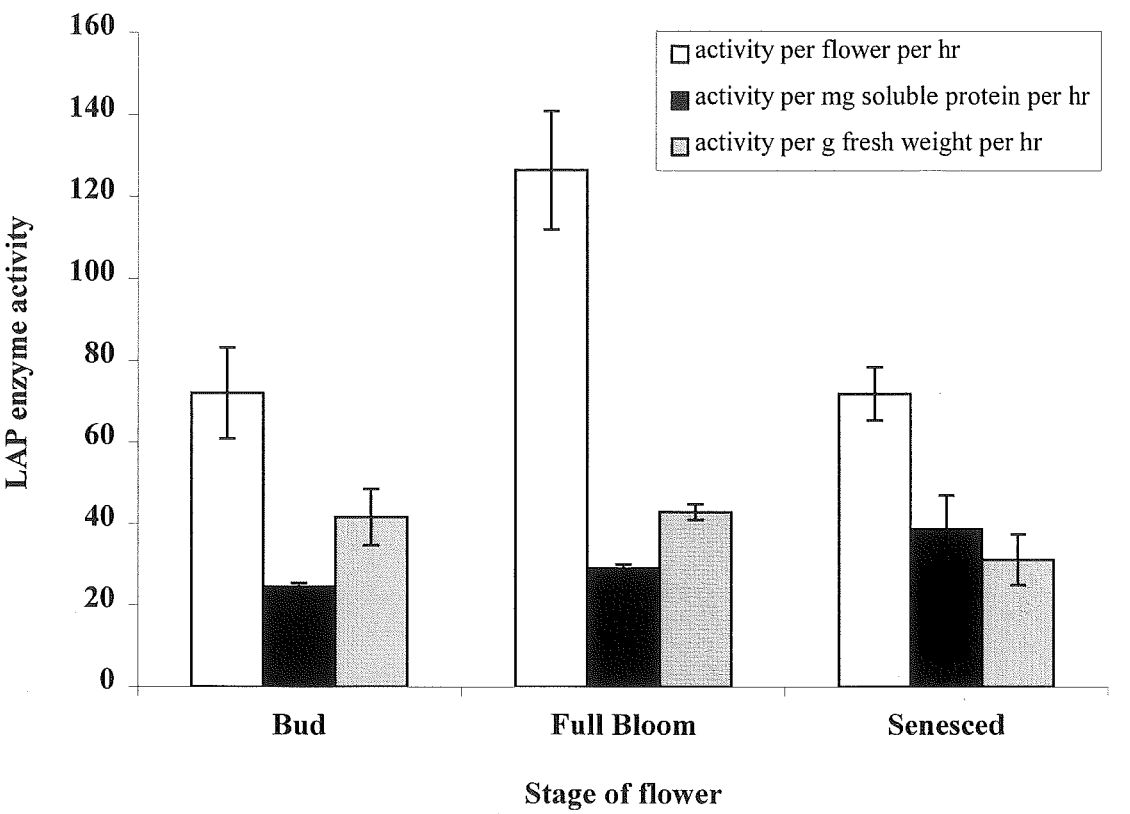


Figure 3.13: LAP activity in petals of cv. Stella D'oro expressed on the basis of three different parameters. Bud: buds collected on the day before opening; Full Bloom: fully open flowers collected on the day of opening; 'Senesced': wilted flowers collected on the day after opening. One unit of enzyme activity is defined as a change in one unit of absorbance at 410 nm at 37°C. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with each extract.

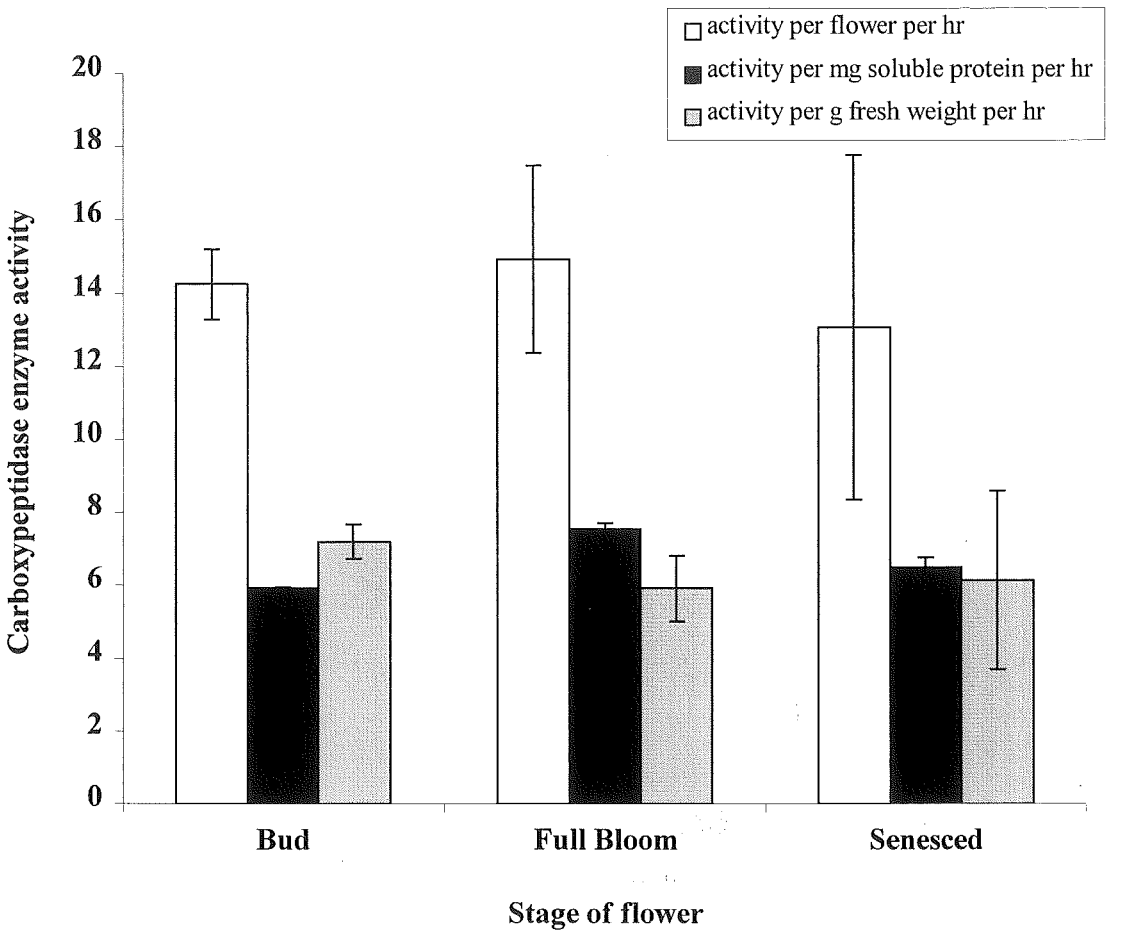


Figure 3.14: Carboxypeptidase activity in petals of cv. Stella D'oro expressed on the basis of three different parameters. Bud: buds collected on the day before opening; Full Bloom: fully open flowers collected on the day of opening; 'Senesced': wilted flowers collected on the day after opening. One unit of enzyme activity is defined as change in mg of amino acids produced at 37°C. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with each extract.

3.4.5 Presence of Endogenous Proteolytic Enzyme Inhibitors or Promoters in Petal Extracts

It is possible that changes in endogenous proteolytic enzyme inhibitors or promoters, if present in daylily petals, could influence the patterns of proteolytic enzyme development therein. Testing for endogenous inhibitors or promoters was carried out by mixing enzyme extracts from petals of 2 different stages of flower development and comparing the enzyme activity of the mixture with that of a calculated theoretical value. If one extract contains an inhibitor or a promoter, it should exert its effect, at least to some extent, on the other extract to produce a set pattern of inhibition or promotion among them. The values obtained for the % inhibition or promotion on endopeptidase, LAP and carboxypeptidase enzymes do not show any set pattern (Table 3.16). The two separate experiments carried out for endopeptidase and LAP enzymes yielded variable results and sometimes completely opposite patterns. Two factor ANOVA without replication for these two enzymes indicated that there was no significant difference ($p>0.05$) between the two different experiments as well as different mixtures of enzymes. Hence, there is no evidence for the presence of endogenous inhibitors or promoters in these extracts.

3.4.6 Low pH Extractable Proteolytic Enzymes in Daylily cv. Stella D'oro

It has been suggested that some of the proteolytic enzymes in various plant tissues are pathogenesis-related ('PR') proteins. A common characteristic of 'PR' proteins is that they are extractable with a low pH buffer. To test whether there are low pH extractable proteolytic enzymes in daylilies, proteins from buds and flowers collected at 3 different stages, as well as leaves and roots of cv. Stella D'oro were extracted in citrate phosphate buffer at pH 2.8 and those extracted with buffers at pH 7 were used

for comparison. Buds (-24 h), full bloom flowers (0 h), senesced flowers (24 h) and roots had undetectable levels of LAP, endopeptidase and carboxypeptidase activities when proteins were extracted in the low pH buffer, even when the enzyme assays were carried out at the standard pHs. Similarly low pH extractable activity of LAP and endopeptidase in leaves was also undetectable. However, low pH buffer extractable carboxypeptidase activity was present in the leaves of cv. Stella D'oro (Table 3.17). The results show that the specific carboxypeptidase activity per mg total proteins extractable with the low pH buffer was 414% of that extracted at pH 7. When expressed on the basis of fresh weight of leaves, low pH-extractable carboxypeptidase activity was 35% of that extracted at pH 7.

3.4.7 LAP

3.4.7.1 LAPs in Different Cultivars of Daylily

Here the data show that the LAP activity in the flowers of daylily cv. Stella D'oro seems to be under developmental control. Flowers of five other daylily cultivars at three developmental stages with clearly discernible visible changes were chosen to confirm and extend this observation. The three stages are: Flower buds collected 2 days before opening, fully open flowers collected on the day of opening and senesced flowers collected 24 h later. All the cultivars except cv. Dallas Star show a similar pattern of LAP activity during opening and senescence (Figure 3.15). Buds had a lower activity while fully open flowers had a higher activity. With flower senescence the activity droped again. However, in the cv. Dallas Star the enzyme activity remained constant during flower development and senescence. Hence, the cv. Dallas Star is different from other cultivars in this respect.

Table 3.16: Effect of mixing enzyme extracts from petals of different stages of flower development on proteolytic enzyme activity.

Stages of enzyme extracts mixed	% inhibition or promotion				
	Endopeptidase		LAP		Carboxypeptidase
Bud (-24 h) × Full Bloom (0 h)	-7.9,	-67.6	-5.2,	-7.4	-1.3
Bud (-24 h) × Full Bloom (6 h)	+28.4,	-15.6	-1.6,	-5.4	+39.4
Bud (-24 h) × Full Bloom (12 h)	+30,	-22.3	+1.8,	+149.3	-4.6
Bud (-24 h) × 'Senesced' (24 h)	+3.2,	-285.5	-15.9,	+12.0	-13.7
Full Bloom (0 h) × Full Bloom (6 h)	-13.0,	-71.7	-1.2,	+42.0	-0.2
Full Bloom (0 h) × Full Bloom (12 h)	+9.2,	-39.7	+4.1,	+49.1	+5.2
Full Bloom (0 h) × 'Senesced' (24 h)	-1.1,	+6.8	-5.2,	+5.1	+14.6
Full Bloom (6 h) × Full Bloom (12 h)	-5.6,	+2.0	-8.0,	+10.7	-61.2
Full Bloom (6 h) × 'Senesced' (24 h)	-5.7,	-13.2	-10.0,	-4.7	+21.6
Full Bloom (12 h) × 'Senesced' (24 h)	+3.3,	-34.1	+4.3,	-11.5	+9.7

Bud: buds collected at 9 a.m. on the day before full bloom (-24 h); Full Bloom: fully open flowers collected at 9 a.m., 3 p. m. and 9 p. m. on the day of opening (0 h, 6 h and 12 h respectively); 'Senesced': fully wilteded flowers collected at 9 a.m. on the day after full bloom (24 h) of cv. Stella D'oro. The two sets of values shown for endopeptidase and LAP enzymes are from two different experiments. Negative values indicate inhibition of enzyme activity while positive values indicate promotion of enzyme activity. Two factor ANOVA without replication for endopeptidase and LAP showed no significant difference ($p>0.05$) between the two different experiments as well as different mixtures of enzymes.

Table 3.17: Low pH extractable carboxypeptidase enzyme activity from leaves of cv. Stella D'oro.

pH of the extraction buffer	**mg amino acids released per h per mg total proteins**	**mg amino acids released per h per g fresh weight of tissue**	**mg total soluble proteins per extract**
7	11.7 ± 0.5	41.2 ± 0.5	5.1 ± 0.1
2.8	48.7 ± 4.8	14.5 ± 1.4	0.4 ± 0.001
carboxypeptidase activity extracted at pH 2.8 as a % carboxypeptidase activity extracted at pH 7.	414	35	8

Errors represent maximum error for two separate extracts. Three replicate activity determinations were carried out with each extract.

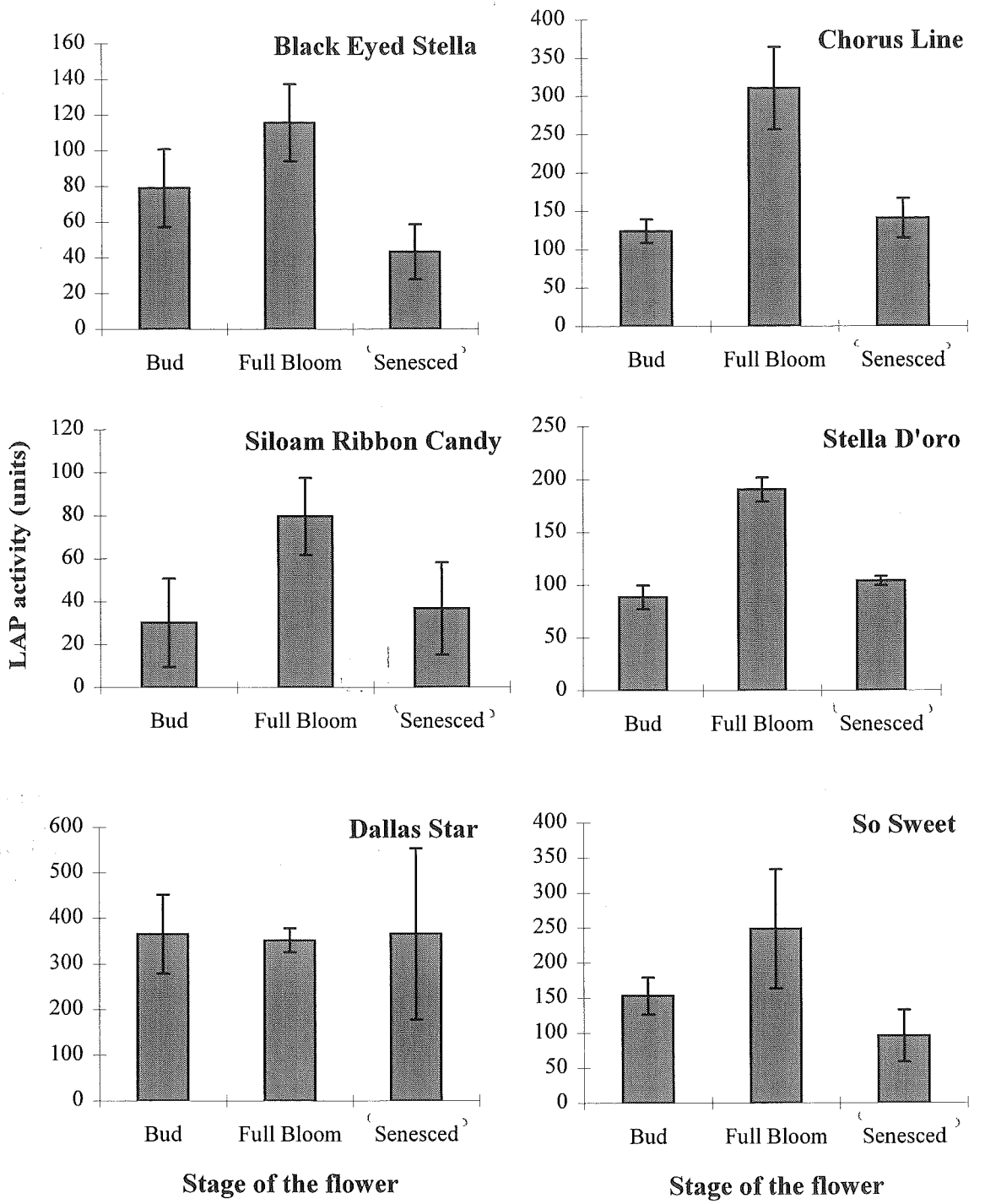


Figure 3.15: Regulation of LAP activity during development of daylily flowers of different cultivars. Bud: buds collected 2 days before opening; Full Bloom: fully open flowers collected on the day of opening; 'Senesced': wilted flowers collected on the day after opening. One unit of enzyme activity is defined as a change in one unit of absorbance at 410 nm per h at 37°C per flower. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with each extract.

3.4.7.2 LAP Isozymes of cv. Stella D'oro

IEF gels revealed that daylily (cv. Stella D'oro) buds, full bloom flowers, and leaves had two different isozyme bands that were close to each other (Plate 3.9A and B). On the other hand senesced flowers and roots appear to have one broad band. It is not clear whether there was only one band or whether there were two bands that were somehow too diffuse for clear separation. Since the bands were very closely positioned in the gel, with a pI value of about 5 it was impossible to determine their pI values separately.

3.4.7.3 Distribution of LAP activity in Daylily Flowers of cv. Stella D'oro

The data about LAP activity in this study had been obtained by extracting all the petal tissues including the tube of different buds or flowers. Furthermore, it was of interest to determine whether there were differences in the LAP activity among inner petals, outer petals and tube of full bloom flowers. Of the total activity, about 41% and 51% were confined to the outer petals and inner petals, respectively, while only 8% was found in the tube (Figure 3.16). Analysis by IEF gel depicts that 2 isozyme bands were present in both outer and inner petals (Plate 3.10A). Similarly, buds also had similar levels of LAP activities in both inner and outer petals; inner petals having a value of 24.2 ± 10 absorbance units per h per flower and outer petals having a value of 19.3 ± 4.1 absorbance units per h per flower. IEF gel of bud extracts (Plate 3.10B) also shows two isozyme bands in both inner and outer petals.

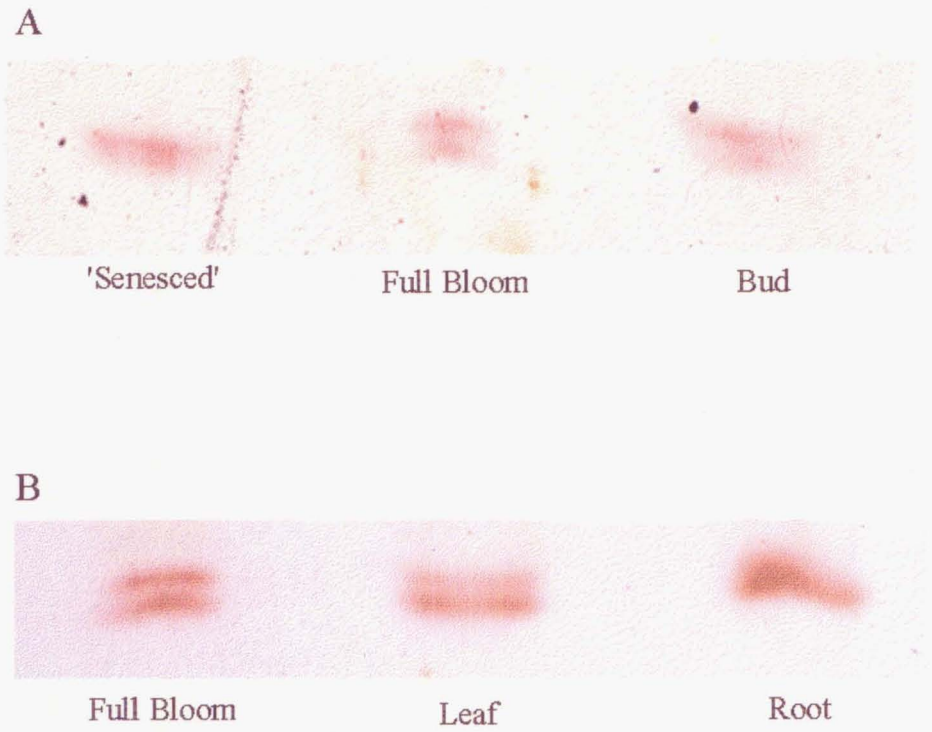


Plate 3.9
IEF gel electrophoresis for LAP isozymes of cv. Stella D'oro. A: LAP isozymes in petals at different developmental stages of flower. Each lane was loaded with 0.03 mg of total proteins. B: LAP isozymes in different parts of the daylily plant. Each lane was loaded with 0.05 mg of total proteins. Bud: bud collected on the day before opening; Full Bloom: fully open flower collected on the day of opening; 'Senesced': wilted flower collected on the day after opening.

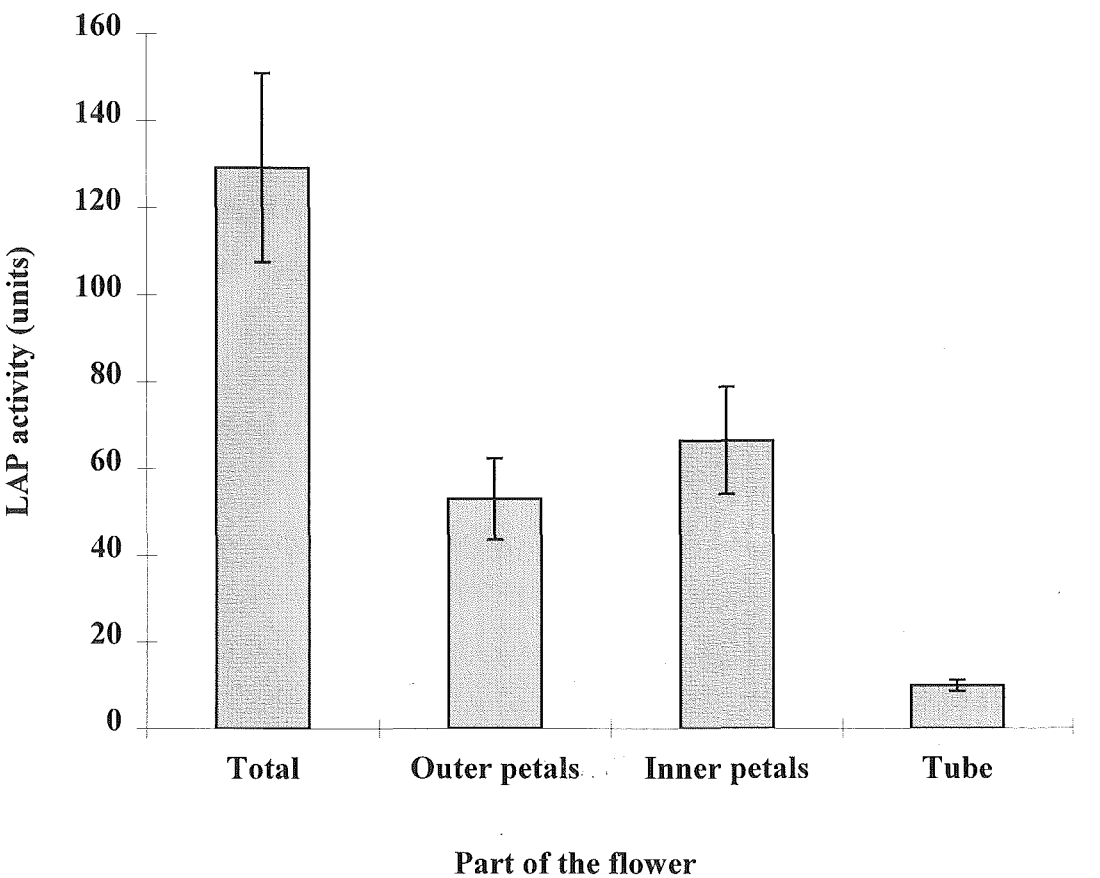


Figure 3.16: Distribution of LAP activity among inner petals, outer petals and tube of fully open flowers of cv. Stella D'oro. One unit of enzyme activity is defined as a change in one unit of absorbance at 410 nm per h at 37°C per flower. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with each extract.

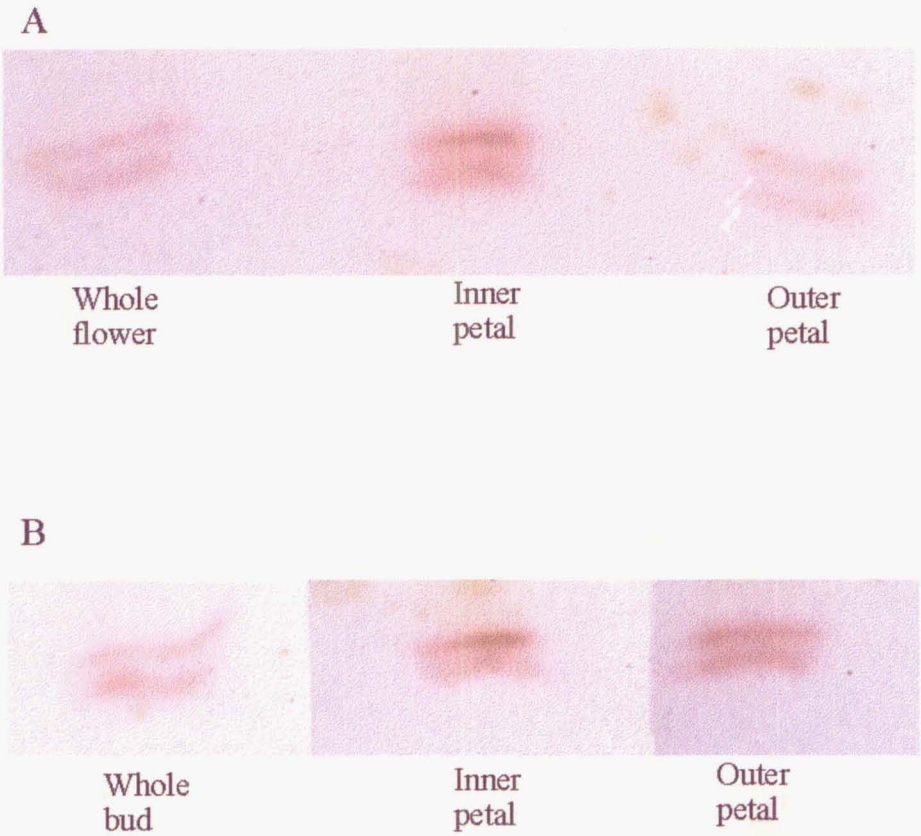


Plate 3.10
IEF gels showing LAP isozymes in inner and outer petals of cv. Stella D'oro. A: extracts prepared from fully open flowers collected on the day of opening. B: extracts prepared from buds collected on the day before opening. Extracts of whole flower and whole bud which consist of inner petals, outer petals and tube were included for comparison. Each lane was loaded with 0.05 mg of total proteins.

3.4.7.4 Effect of Damaging Floral Parts on LAP Activity in Daylily Petals of cv. Stella D'oro

In tomato, LAP activity was inducible by wounding. Here, this possibility was investigated with daylily flowers. Different floral parts were deliberately damaged on the day before flower opening and the LAP activity in the petals was then analysed on the day of opening to study the effect of damaging on the enzyme activity in the petals. As shown in Figure 3.17, damaging floral parts did not have any effect on the enzyme activity of the daylily petals.

3.4.7.5 Partial Purification of LAP from Petals of cv. Stella Doro

It was of interest to seek evidence that LAP and endopeptidase activities in dayliliy flower extracts were contributed by distinct enzyme proteins. Partial purification of LAP enzyme had been performed using petals of full bloom flowers of cv. Stella D'oro. The crude extract was subjected to ammonium sulphate precipitation, dialysed and concentrated by PEG-20,000 and loaded onto a DEAE cellulose column giving rise to different fractions, some of which were pooled and concentrated by PEG-20,000. The elution profile of the DEAE cellulose column is shown in Figure 3.18. It shows a major peak of LAP activity in the fractions between 12 and 21, indicating that the enzyme had been eluted out from the column with 10 mM sodium phosphate buffer (pH 7) without binding to the column. The fractions with LAP activity units above 48 (between 12 to 19) were pooled for later analysis for endopeptidase enzyme activity and characterization of LAP enzyme. The percentage yield and the purity factor at each step of purification are summarized in Table 3.18. Approximtely only 3-fold purification was obtained at the final step. SDS PAGE and Coomasie blue staining revealed that almost all the bands in the crude extract were also present in the pooled DEAE-column fractions (data not shown). The pooled fractions with high LAP had no detectable level of endopeptidase activity.

3.4.8 Characterisation of LAP from petals of cv. Stella D'oro

3.4.8.1 Effect of High Temperatures on LAP Stability

In the literature, there is some evidence that some LAP enzymes are heat stable. Here, higher LAP activity in the crude extracts was observed when the enzyme assays were carried out at 37 and 50°C than at 60 and 70°C (Table 3.19). Preincubation of the extract at 60 and 70°C even in the presence of Mn^{++} ions (shown to promote LAP activity-see section 3.4.8.2) brought about 90-95% loss of LAP activity.

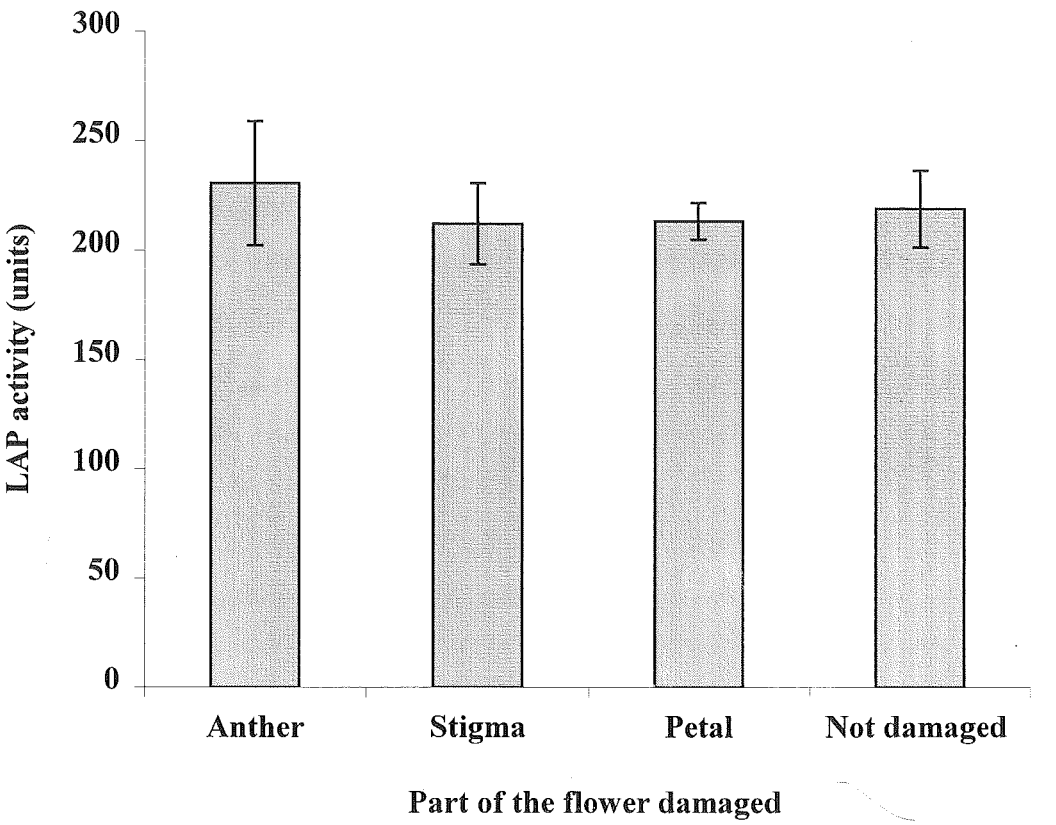


Figure 3.17: Effect of damaging floral parts on LAP activity of petals of cv. Stella D'oro. One unit of enzyme activity is defined as a change in one unit of absorbance at 410 nm per h at 37°C per flower. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with each extract.

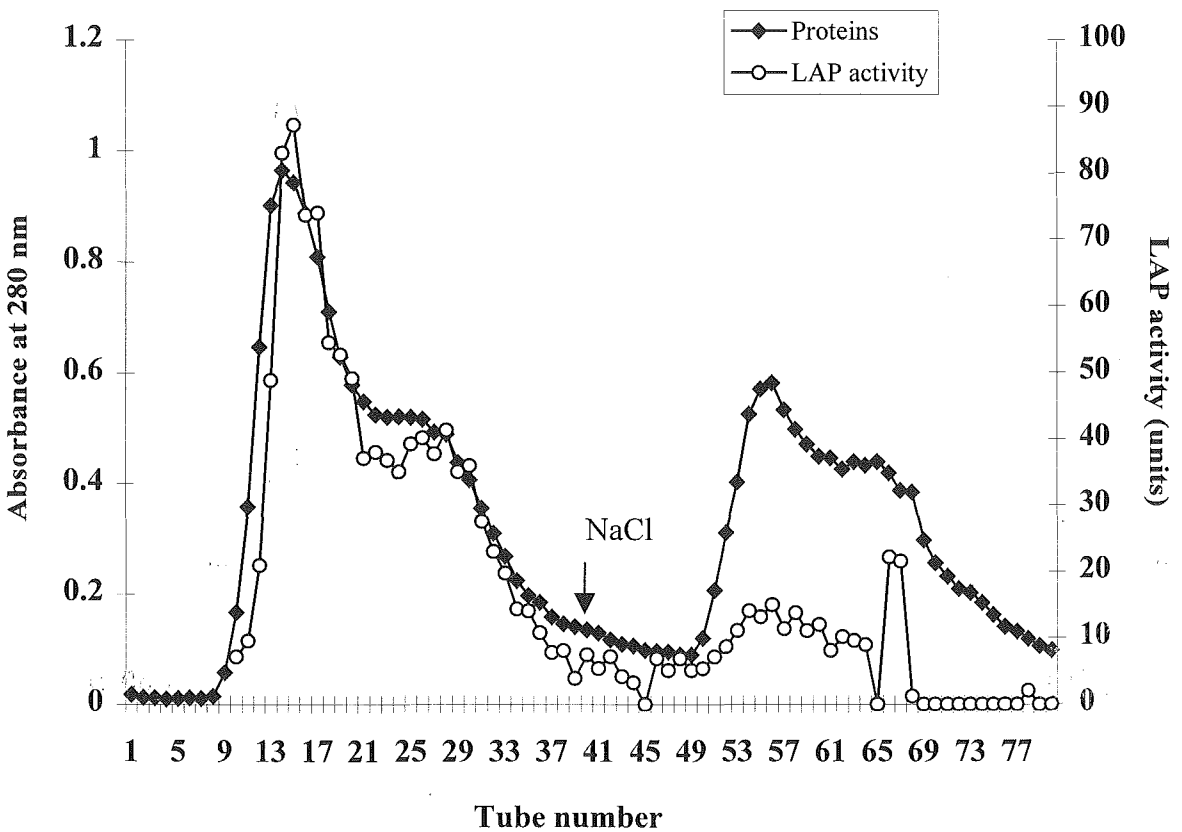


Figure 3.18: Purification of LAP by DEAE cellulose column. The first 40 fractions were eluted with 10 mM phosphate buffer (pH 7), while the next 40 fractions were eluted using 10 mM phosphate buffer (pH 7) containing 0.4 M sodium chloride. One unit of enzyme activity is defined as a change in one unit of absorbance at 410 nm per h at 37°C. Protein content was measured as absorbance at 280 nm.

Table 3.18: Summary of steps in the purification of LAP from petals of full bloom flowers of daylily cv. Stella D'oro.

Step	Volume (ml)	Total LAP activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Yield %	Purity factor (-fold)
Crude	148	2220	363.8	6.1	100	1
$(NH_4)_2SO_4$ 30-60%	17	1105	87	12.7	49.8	2.1
DEAE cellulose column (fractions 12-20)	12	103.2	5.7	18.1	4.6	3.0

One unit of enzyme activity is defined as a change in one unit of absorbance at 410 nm per h at 37°C.

Table 3.19: Effect of temperature on LAP activity.

	LAP activity as a % of activity at 37°C		
Testing temperature	**50°C**	**60°C**	**70°C**
Enzyme activity assayed at the temperature to be tested without preincubation	127.3 ± 20	30 .6 ± 2.6	53.5 ± 20.8
Preincubation at a testing temperature for 5 min before assaying for enzyme activity at 37°C	74.7 ± 6.5	25 ± 2.6	1 ± 1
Preincubation at a testing temperature for 10 min before assaying for enzyme activity at 37°C	63 ± 8.9	13 ± 0	3.7 ± 2.9
Preincubation at a testing temperature for 5 min with 0.5 mM Mn^{++} before assaying for enzyme activity at 37°C	n.d.	9 ± 2	3.3 ± 0.6

n.d.: not determined.

3.4.8.2 Effect of Different Chemicals on LAP Activity in Crude Flower Extracts

Further experiments in characterization of LAP activity were carried out using crude extracts from full bloom flowers of cv. Stella D'oro. Of the chemicals tested, 1 mM $MnSO_4$ increased the LAP activity significantly (Figure 3.19). Other chemicals neither greatly promoted nor inhibited the enzyme activity. Then a detailed experiment was carried out with three different concentrations, namely low (L), medium (M) and high (H) concentrations of some of the chemicals (Figure 3.20). Since iodoacetamide blocks thiol groups in the enzyme and since the need for thiol groups has been tested during optimization of enzyme activity (section 3.4.1) iodoacetamide was not included in the later experiments. Since, bestatin at the concentration tested (0.1 mM) was high enough to inhibit LAPs from other plants, it was also not tested further. Increasing NEM and PMSF concentrations inhibited the enzyme activity proportionally. In contrast, as little as 0.1 mM $MnSO_4$ brought about the promotion of enzyme activity. Leupeptin at the 3 concentrations tested did not have a significant effect on the LAP activity. It should be noted that 10 mM $MnSO_4$, 100 mM $MgSO_4$ and 10 mM or 100 mM $ZnSO_4$ caused precipitation of proteins after mixing with the chemical (data not shown). Enzyme inhibition by these chemicals could not be determined reliably in these cases.

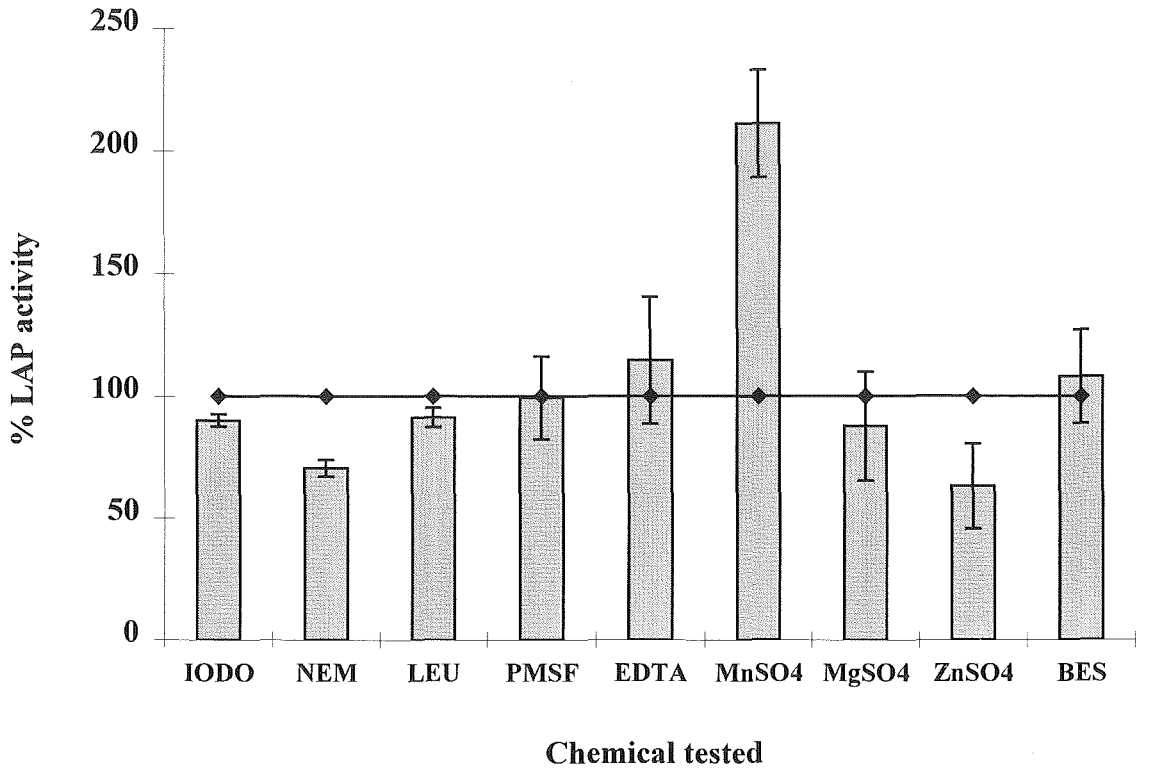


Figure 3.19: Effect of known proteolytic enzyme activators and inhibitors on LAP activity in crude daylily flower extracts. IODO: 1 mM Iodoacetamide; NEM: 1 mM NEM; LEU: 10 μM Leupeptin; PMSF: 1 mM PMSF; EDTA: 1 mM EDTA; $MnSO_4$: 1 mM $MnSO_4.4H_2O$; $MgSO_4$: 1 mM $MgSO_4.7H_2O$; $ZnSO_4$: 1 mM $ZnSO_4.7H_2O$; BES: 0.1 mM Bestatin. Enzyme activity was determined using extracts from full bloom flowers collected on the day of opening. One unit of enzyme activity is defined as a change in one unit of absorbance at 410 nm per h at 37°C. Enzyme activity at 410 nm per h at 37°C in the absence of any chemical is regarded as 100%. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with each extract.

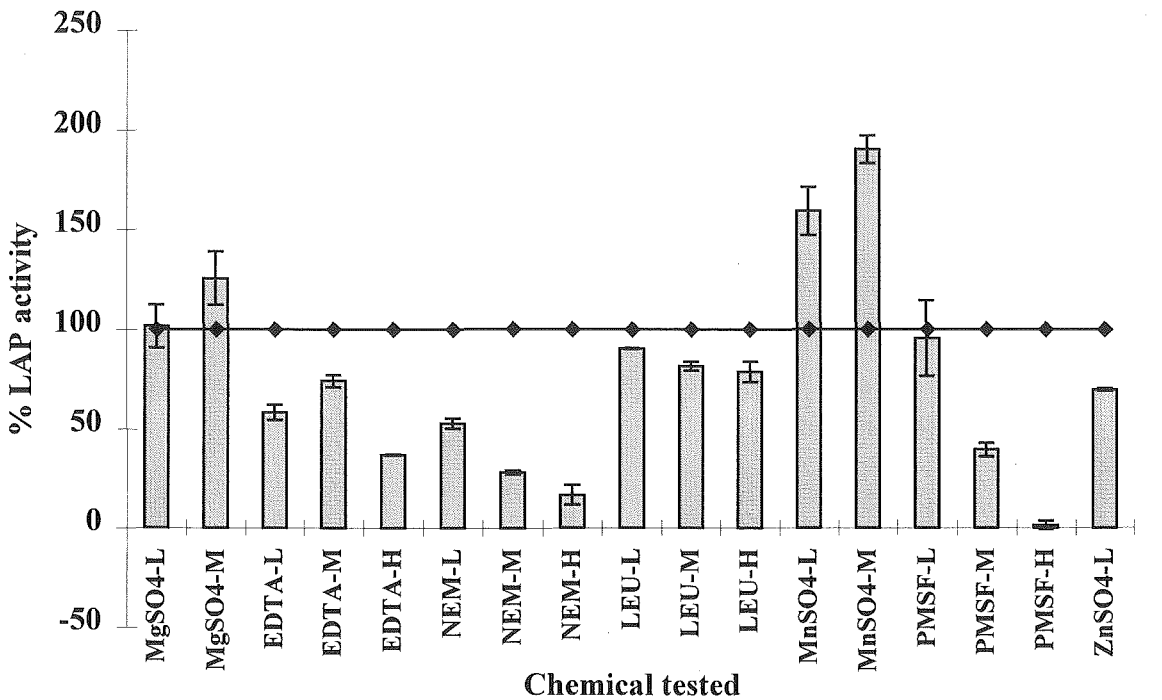


Figure 3.20: Effect of different concentrations of known proteolytic enzyme activators and inhibitors on LAP activity in crude extracts. $MgSO_4$: L=1 mM; M=10 mM; EDTA:L=1 mM; M=10 mM; H=100 mM; NEM: L=1 mM; M=10 mM; H=100 mM; LEU (Leupeptin): L=10 μM; M=100 μM; H=500 μM; $MnSO_4$: L=0.1 mM; M=1 mM; PMSF: L=1 mM; M=2 mM; H=3 mM; $ZnSO_4$: L: 1 mM. Enzyme activity was determined using extracts from full bloom flowers of cv. Stella D'oro collected on the day of opening. One unit of enzyme activity is defined as a change in one unit of absorbance at 410 nm per h at 37°C. Enzyme activity at 410 nm per h at 37°C in the absence of any chemical is regarded as 100%. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with each extract.

3.4.9 LAP Activity in Cut Full Bloom Flowers after 24 hr Treatment with Several Chemicals

Full bloom flowers treated with some of the chemicals described in 2.3.7.7 were harvested 24 h after the treatment to analyse for LAP activity. Flowers that stayed open after treatment with cycloheximide had a significantly higher enzyme activity than those that senesced after treatment with water (Figure 3.21). In contrast, flowers that senesced after treatment with all the other chemicals showed enzyme activity levels similar to those of the senesced flowers in the water control. SDS PAGE analysis showed cycloheximide treated flowers had more protein bands with higher molecular weights than those treated with water (Plate 3.11).

Since none of the chemicals tested except cycloheximide had any effect on flower longevity, the flowers treated with these chemicals were chosen randomly (exept cycloheximide due to its effect in prolonging vase longevity) to analyse for LAP activity.

3.4.10 LAP Activity in Leaf Discs after Treatment with Several Chemicals

Leaf discs treated with some chemicals in relation to senescence were harvested to study their effect on LAP activity in leaves. The chemicals tested were cycloheximide, ethrel, silver thiosulfate and ABA. Cycloheximide and ethrel were selected due to their effect in retaining green colour (see section 3.3.2) and also due to their action as protein synthesis inhibitor and ethylene production respectively. Silver thiosulfate was selected because of its action against ethylene and also because it caused yellowing of leaf discs around the same time as those kept in water. Leaf discs in ABA were analysed because it enhanced yellowing of leaf discs.

Leaf discs in ethrel and cycloheximide that retained green colour until day 14 had very low LAP activity when compared to leaf discs in water control that had turned 50% yellow (Figure 3.22). However, there was no substantial difference in total soluble protein contents in leaf discs treated with cycloheximide and water. Total soluble protein in leaf discs treated with 0.1 mg/L cycloheximide for 7 days was 0.067 ± 0.003 mg/disc (or 10.52 ± 1.03 mg/g fresh weight of tissue) when compared to 0.051 ± 0.019 mg/disc (or 19.07 ± 10.8 mg/g fresh weight of tissue) in discs kept in water for 7 days.

Leaf discs which turned yellow in ABA also had lower LAP activity as opposed to the green leaf discs in water control. On the other hand, leaf discs which turned yellow in silver thiosulfate had LAP activities similar to those yellow ones kept in water.

No difference in LAP activity was detected in green leaf discs before incubation, those kept in water for 2 days (green) and for 11 days (50% yellow) (Table 3.20). Similarly there was no dramatic change in soluble protein contents during incubation in water.

The LAP activities in leaves harvested from the glasshouse-grown daylily plants of cv. Stella D'oro at different stages of development are shown in Figure 3.23. There was an increase in LAP activity as leaves mature, but remained unchanged when they turned yellow. The senesced leaves were probably at some different undefined stages of maturity, hence giving rise to the large error.

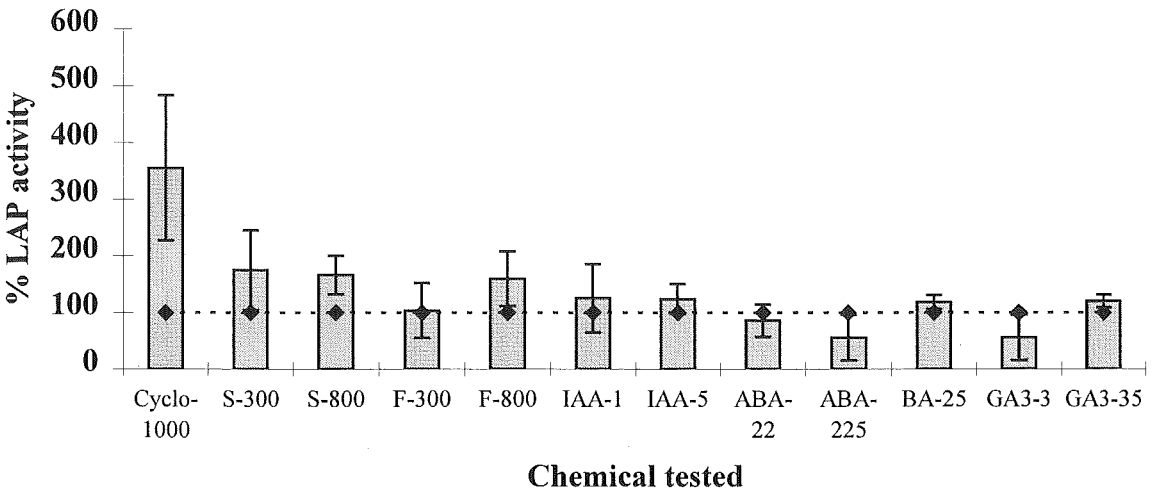


Figure 3.21: Effect of different chemicals on the level of LAP activity in the petals of daylily flowers at pH 7 following 24 h of immersing the cut ends of the pedicels of full bloom flowers into the test solution. Chemicals used are cyclo-1000: 1000 mg/L cycloheximide; S-300: 300 mg/L sucrose; S-800: 800 mg/L sucrose; F-300: 300 mg/L fructose; F-800: 800 mg/L fructose; IAA-1: 1 mg/L IAA; IAA-5: 5 mg/L IAA; ABA-22: 22.5 mg/L ABA; ABA-225: 225 mg/L ABA; BA-25: 25 mg/L BA; GA3-3: 3.5 mg/L GA_3; GA3-35: 35 mg/L GA_3. Enzyme activity in the petals following 24 h of dipping in water (water control) was taken as 100% (the dotted line). One unit of enzyme activity is defined as change in units of absorbance at 410 nm per h per flower. [Error bars represent standard deviation for three flower extracts.]

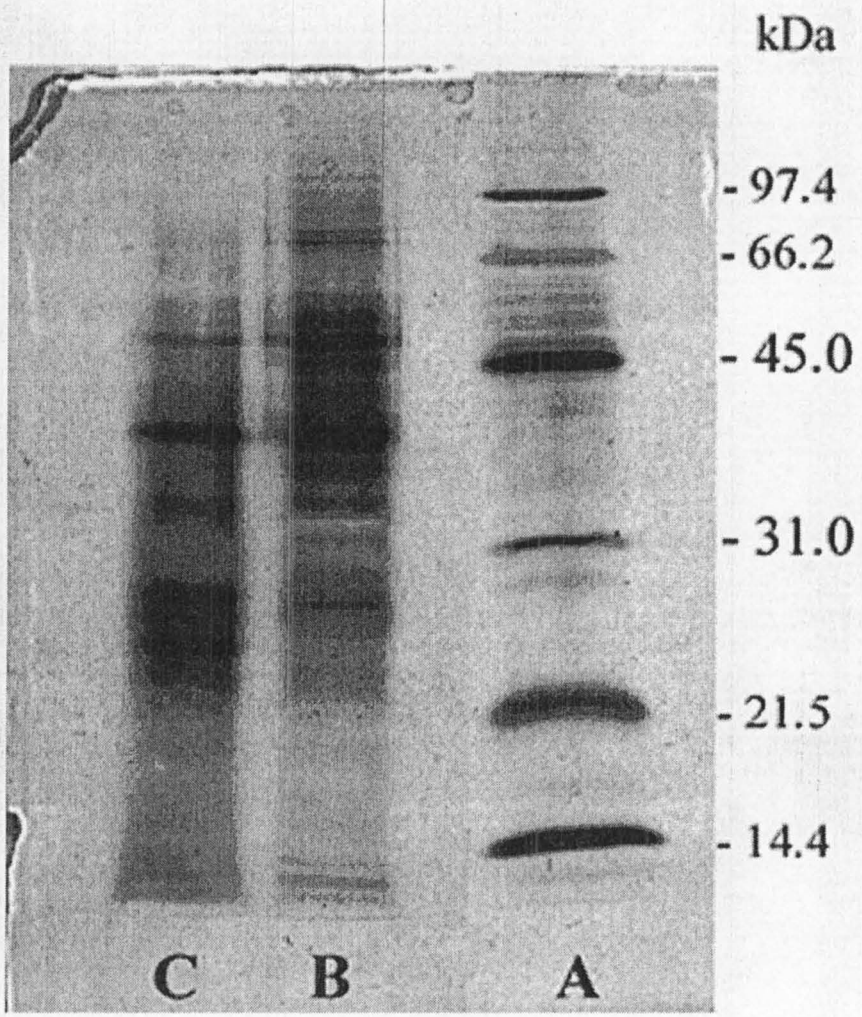


Plate 3.11
SDS PAGE of soluble proteins extracted from full bloom flowers of cv. Stella D'oro, 24 h after treatment with water and cycloheximide. A: molecular weight standards; B: cycloheximide (1 mg/mL); C: water (control). Proteins were extracted in 0.01 M phosphate buffer at pH 7. The gel was subjected to silver staining after electrophoresis.

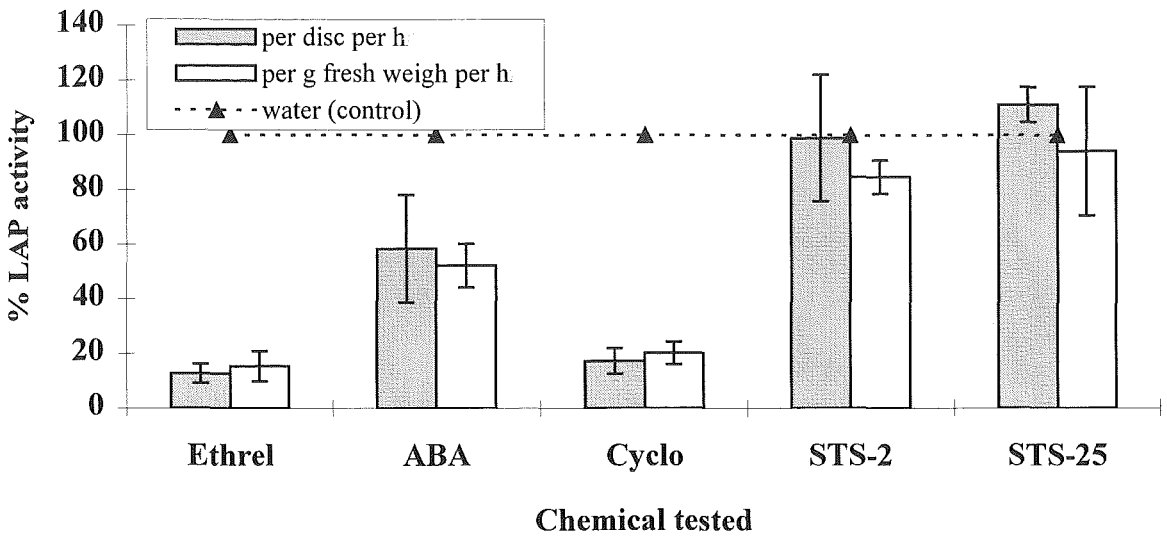


Figure 3.22: Effect of different chemicals on LAP activity of leaf discs of cv. Stella D'oro. Ethrel: 1.4 mg/L ethrel; ABA: 22.5 mg/L ABA; Cyclo: 0.1 mg/L cycloheximide; STS-2: 2.5 mg/L silver thiosulfate; STS-25: 25 mg/L silver thiosulfate. Leaf discs in ethrel and ABA had been harvested on 11th day and 2nd day after treatment, respectively while those in cycloheximide and silver thiosulfate had been harvested on 7th day after treatment. Enzyme activity in leaf discs from each chemical treatment had been compared with leaf discs treated with water and harvested on the same day. Harvesting had been carried out when 50% of leaf discs in water or in the chemical turned yellow. Enzyme activity in the leaf discs following incubating in water (water control) was taken as 100% (the dotted line). One unit of enzyme activity is defined as change in units of absorbance at 410 nm per g fresh weight per h or per disc per h. [Error bars represent standard deviation for three separate extracts each with 2 discs.]

Table 3.20: LAP activity and soluble proteins in cv. Stella D'oro leaf discs. One unit of enzyme activity is defined as units of absorbance at 410 nm. [Errors represent standard deviation for three separate extracts each with 2 discs.]

Tissue type	**LAP activity (units)**		**Soluble proteins (mg)**	
	per disc per h	**per g fresh weight of tissue**	**per disc**	**per g fresh weight of tissue**
Before incubation (green)	1.15 ± 0.17	191.7 ± 30.8	0.035 ± 0.004	5.91 ± 0.9
Incubated in water for 2 days (green)	1.29 ± 0.21	193.9 ± 22.6	not determined.	not determined
Incubated in water for 11 days (50% yellow)	1.22 ± 0.24	172.3 ± 42.1	0.029 ± 0.01	4.45 ± 1.8

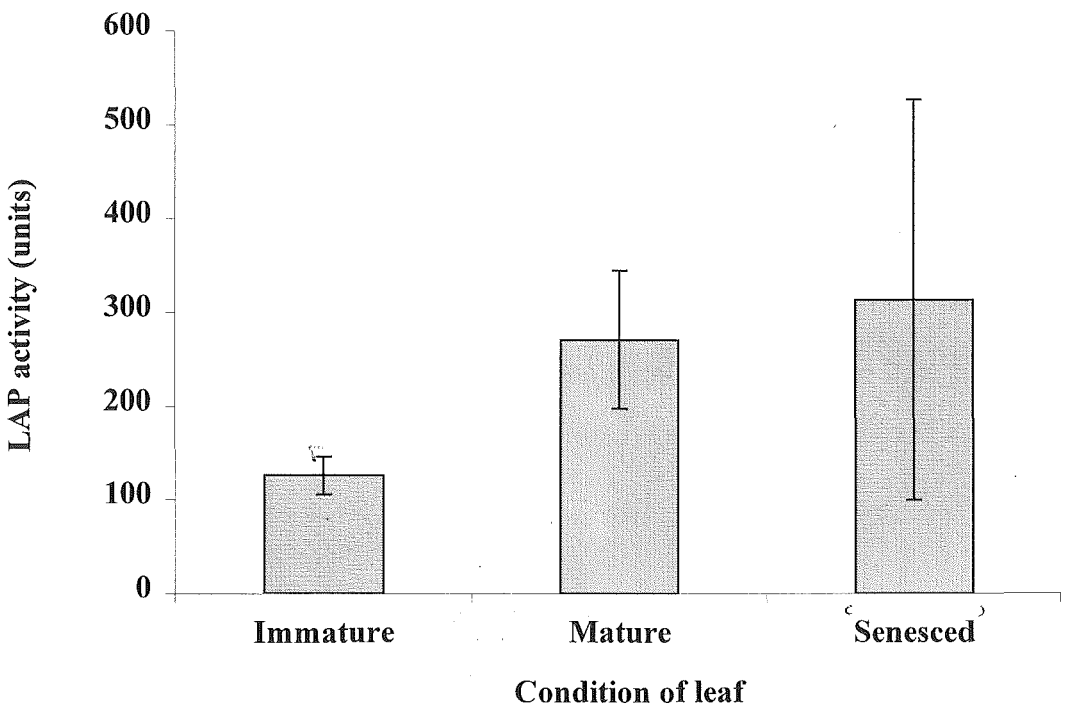


Figure 3.23: LAP activity of leaves from cv. Stella D'oro grown in glasshouse. Immature: soft, green leaves of about 8-10 cm long and collected from the centre of the crown; Mature: rough, green leaves of about 40 cm long and collected from the periphery of the crown; 'Senesced': rough, yellow leaves of about 40 cm long and collected from the periphery of the crown. One unit of enzyme activity is defined as change in units of absorbance at 410 nm per h per g fresh weight. [Error bars represent standard deviation for three flower extracts.]

3.4.11 Regulation of LAP in daylily flowers, cv. Stella D'oro at the nucleic acid level: a preliminary study

3.4.11.1 Probe Preparation

The data in this study has indicated that LAP in daylily petals seems to be under developmental regulation. It was of interest to initiate a study of this enzyme at the molecular level. And the *lap* cDNA probe from *Arabidopsis thaliana* as described by Bartling and Weiler (1992) was used as a heterologous probe in this study.

Concentration of the probe had been estimated by comparing a series of dot blots of the probe with a similar series of a DIG-labelled control DNA of known concentration. Dots were compared visually, based on the intensity of the colour and the diameter. It appears that the dot produced by the ×10 diluted probe falls between the dots produced by 0.1 and 0.2 pg/μL of the control DNA (Plate 3.12). This means the concentration of the probe should be between 1 and 2 pg/μL (1-2 μg/mL). The amount of labelled probe to be used in RNA dot blot hybridization had been determined based on this value.

When genomic DNA isolated from petals of full bloom flowers and leaves of cv. Stella D'oro were subjected to dot blot hybridization with the *lap* cDNA probe, a visible dot was produced only by genomic DNA from leaves (results not shown). Production of a dot by leaf DNA confirmed *lap* cDNA from *A. thialiana* was suitable for use in heterologous hybridization experiments with daylily cv. Stella D'oro.

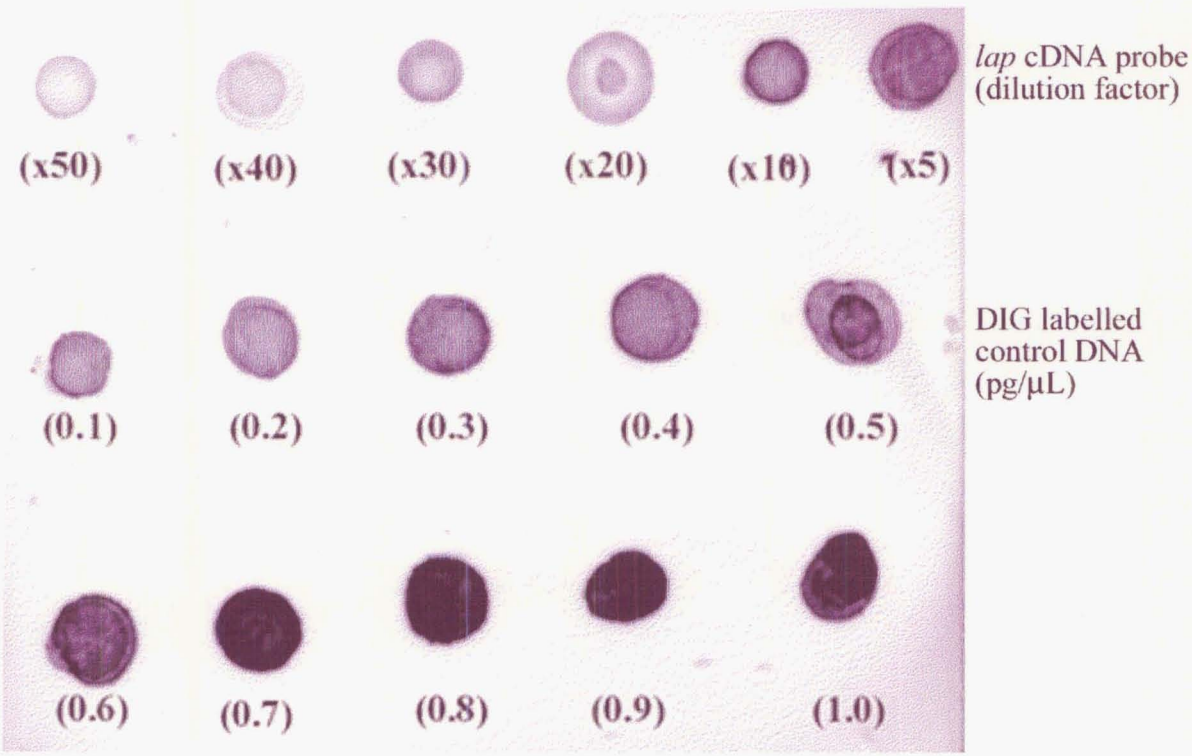


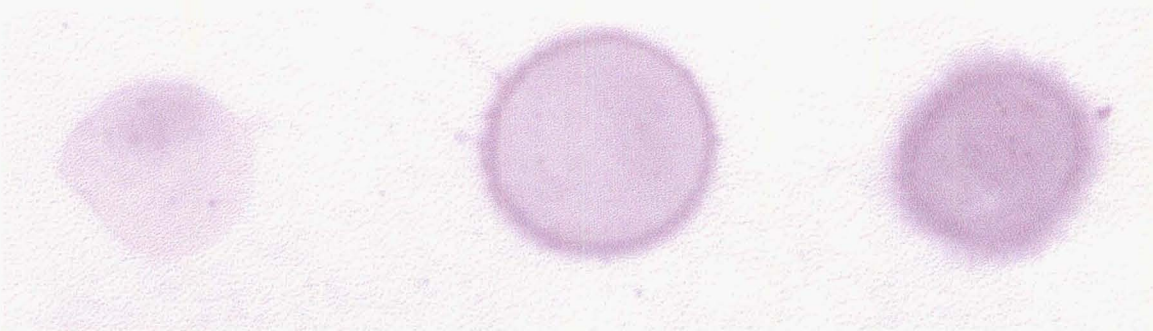
Plate 3.12
Quantitation of *lap* cDNA.

3.4.11.2 RNA Dot Blot Hybridization

RNA dot blotting was carried out with 1, 2 and 3 μg of total RNA from buds, full bloom and senesced flowers of cv. Stella D'oro to find out the best amount of RNA to be spotted to get a good signal (data not shown). It appeared that 1 or 2 μg of total RNA is suitable for dot blot analysis.

The *lap* mRNA levels per 1 μg total RNA appear to remain constant in buds and full bloom flowers. It was very low in the senesced flower (Plate 3.13A). RNA extracted from inner and outer petals of buds and full bloom flowers showed that *lap* mRNA is distributed in equal proportion among inner and outer petals (data not shown). Level of *lap* mRNA in cycloheximide-treated flower was slightly higher than that of water-treated flower, but considerably lower than that of fully open flower (Plate 3.13B).

A



B

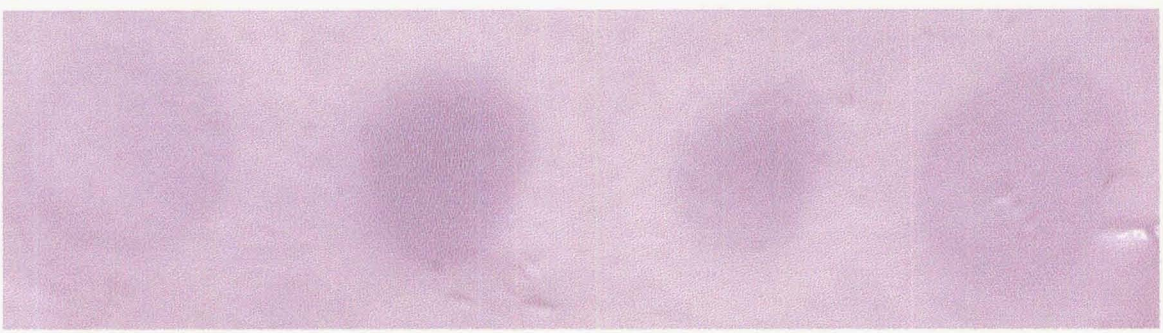


Plate 3.13
RNA dot blots carried out on total RNA extracted from petals of daylily cv. Stella D'oro hybridized with a *lap* probe from *Arabidopsis thaliana*. A: RNA from different developmental stages of flower. Each dot had 1 µg of total RNA from daylily flowers. B: Effect of cycloheximide on flower-*lap* mRNA. Each dot had 2 µg of total RNA from daylily flowers. Bud: bud collected on the day before opening; Full Bloom: fully open flower collected on the day of opening; 'Senesced': wilted flower collected on the day after opening; Cycloheximide: fully open flower treated with cycloheximide (1 mg/mL) for 24 h; Water: fully open flower treated with water for 24 h.

CHAPTER 4

DISCUSSION

4.1 MICROPROPAGATION

4.1.1 Optimum Conditions for the Production of Daylily Plantlets

In micropropagation of daylily by tissue culture, floral explants from unopened buds appear to be the most used explant type, although shoot tips (Strode and Oglesby, 1976; Smith and Krikorian, 1991) and flower inflorescences (Meyer, 1976) have also been used successfully. However, use of explants from tuberous roots (Mullin, 1970; Heuser and Apps, 1976) and flower stalks (Heuser and Apps, 1976) did not result in plantlets. Use of buds has the advantage since they can be obtained in large numbers and the parent plant from which the buds were obtained does not have to be destroyed (Krikorian et al., 1990) as in the case of shoot apex. Of the buds, ovaries (Krikorian and Kann, 1977; Krikorian et al., 1995) and petals (Chen and Holden 1972; Apps and Heuser, 1975; Heuser and Apps, 1976) are the preferred explant type. In the present study, both petal and ovary explants from small- and medium-sized buds were used for the initiation of shoot primordia-like structures. On average ovary explants appear to be better than petal explants in producing shoot primordia-like structures in all the cultivars tested except in the cv. Stella D'oro where the response from ovary explants was very poor.

The effect of the size of the bud in producing shoot primordia-like structures depends on the cultivar. Except ovary explants from the cv. Dallas Star, all the other explants from both small- and medium-sized buds responded more or less the same way. In the cv. Dallas Star, ovary explants from small-sized buds were better in producing shoot primordia-like structures than those from medium-sized buds.

Heuser and Apps (1976) reported that in producing callus from flower buds of cv. Chipper Cherry, there was no callus formation from buds smaller than 0.5 cm in length. According to them, callus was formed only from large (1 cm) flower buds.

The experiment on the effect of position of the petal explant on initiation of shoot primordia-like structures produced interesting results. Only the explants from base of the petals and tubes produced shoot primordia-like structures. This could be due to the presence of actively dividing cells in the base of the petals and the tubes. A similar phenomenon has been observed with lily leaves where the explants from bases of the scale leaves showed a higher regeneration capacity when compared to very low and no regeneration in explants from middle and apical parts (Robb, 1957).

Generally, adventitious shoots are formed in the presence of higher concentrations of cytokinins. In daylily variety 'Azetec Gold' multiple shoots were produced when IAA (0.2 mg/L) and 2iP (25 mg/L) were supplemented in the medium (Strode and Oglesby, 1976). Similarly, 2,4-D (1 mg/L) and kinetin (1 mg/L) had been used for the initiation of shoots on *H. flava* callus. The results obtained from this study indicate that other cytokinins and auxins such as 2iP and NAA in certain combinations can also bring about a good shoot primordia forming response.

In vitro shoots often form roots in the presence of auxins such as IAA, IBA or NAA, or in the absence of any growth regulators (Dodds and Roberts, 1985). In this research, root initiation and elongation could occur in growth regulator free-media. Although it was assumed that externally added growth regulators carried over with to shoot primordia-like structures in the shooting medium would disappear with subculturing and would not have an effect on rooting, the results indicate that this is not the case. The conditions provided for the initiation of shoot primordia-like structures appear to have an effect on subsequent rooting and plantlet production. For example, ovary explants from 2 cm long buds of cv. Siloam Ribbon Candy showed a very good response (between 76-100%) in producing shoot primordia-like

structures in all the media tested. However, only the primordia-like structures from ovary explants that were grown in media containing 5 mg/L NAA and 1 mg/L BA and 3 mg/L NAA and 3 mg/L BA produced roots in good numbers. When auxins are required, root formation generally takes place in media with a relatively high auxin and low cytokinin concentration (Hussey, 1983; Dodds and Roberts, 1985). In *H. flava*, multiple adventitious roots were formed from petal explants when grown in high concentrations (6 mg/L as opposed to 2 and 4 mg/L) of NAA in the dark (Chen and Holden, 1972). Rooting of shoot tip cultures of variety 'Azetec Gold' has been induced by 10 mg/L IAA and 2 mg/L NAA in the medium with added NaH_2PO_4 (Strode and Oglesby, 1976). With the conditions used in this research, it is difficult to come to a conclusion about the auxin to cytokinin ratio for root induction in the cultivars tested. However, it is interesting to mention that all the explants that gave rise to more than 20 plantlets in rooting medium were originally grown in the presence of NAA and BA. Explants grown in the presence of NAA and 2iP did not produce plantlets in large numbers.

From the results it is clear that there is no one medium or an explant type can be described as the best for all the cultivars tested. However, there can be an interaction among cultivar, medium constituents and tissue type (Heuser and Apps, 1976); hence the selection of explant, media and growth regulators could vary among species, cultivars and clones. The best medium for the production of shoot primordia-like structures may not be the best for rooting and later development of plants. In cv. Siloam Ribbon Candy highest number of plantlets resulted from shoot primordia-like structures induced from ovary explants in media with combinations 5 mg/L NAA and 1 mg/L BA and 3 mg/L NAA and 3 mg/L BA for buds of both sizes. Hence, ovary explants and the above two media could be used in the future for further development of the micropropagation technique for this cultivar. Unfortunately, for other cultivars, it is not possible to make definite conclusions about the best combination of explant and the medium. In the cv. Chorus Line, although ovaries were better in producing shoot primordia-like structures, they were

not very good in producing plantlets, except the ones grown in 1 mg/L NAA and 5 mg/L BA. This could be due to the requirement of growth regulators for rooting. The same could be true for the cv. Dallas Star where there was production of shoot primordia-like structures to some extent, but no further development into plantlets. In the case of cv. Stella D'oro, a satisfactory number of shoot primordia-like structures were not produced by any of the explant types tested. But the shoot primordia-like structures developed on the growth regulator combination of 3 mg/L each of NAA and BA initiated a considerable number of rooted plantlets. Therefore, further investigations on the best explant, growth regulator combination and growth media are needed for the cvs. Chorus Line, Dallas Star and Stella D'oro. In addition to the factors studied here, there are many other factors that influence organogenesis *in vitro*. These include concentration and source of nitrogen and carbon, pH of the medium, physical condition of culture and the condition of light. Besides, the condition of the parent plant such as the age, physical state and health, and even the age of the tissue or organ from which the explant have been obtained could have an effect on the success of the micropropagation protocol (Jones, 1983; Dodds and Roberts, 1985). To optimise the micropropagation technique these factors might also have to be taken into consideration.

Although it is well established that sugar is necessary for adventitious root formation (Dodds and Roberts, 1985), it does not seem to be true for daylily cultivars tested. There is no effect of sucrose on root formation in the daylily cultivars tested.

In the literature it has been reported that the use of growth regulators such as 2,4-D, NAA and synthetic cytokinins such as BA could lead to an increase in the number of mutations (Hussey, 1983; Jones, 1983). In daylily the genetic stability of plants regenerated via tissue culture methods appear to be dependent on the type and age of the culture. In *H. flava* there was no chromosome variation in the callus tissue grown in the media containing kinetin and 2,4-D for 30 months, or in the plantlets derived

from those callus tissues (Chen and Holden, 1972; Goeden and Chen, 1978). There was no change in ploidy and chromosome morphology in the plants obtained from liquid cultures (Krikorian et al., 1982). Plants obtained from small granules of such suspension cultures were cytologically homogeneous and were identical to the chromosomes of the plant from which the initial explant was taken (Krikorian et al., 1982). Detailed studies on karyotypes of plants derived from suspension cultures of cv. Autumn Blaze that produced embryonid forms showed that there was an increase in diversity of karyotype of plants with an increase in the age of liquid culture (Krikorian et al., 1982). There was translocation of a fragment of chromosome from the short homologue pair of ten to long homologue pair of three. In addition, there was development of tetraploids and aneuploids (2n+1).

Plants derived by tissue culture in this research were very similar in morphology and behaviour to the plants from which the explants had been obtained. Testing for the genetic variability of the plants generated by tissue culture has not been attempted in this research. According to previous literature on daylily, genetic variations of *in vitro* plantlets have been observed only in those regenerated from cell and protoplast suspension cultures (Krikorian et al., 1982) but not in plantlets obtained from callus (Chen and Holden, 1972; Goeden and Chen, 1978). Therefore it is logical to assume that the plants regenerated in this research are genetically equivalent to parent plants.

4.1.2 Success of Tissue Culture in this Research and Futher Applications

The main objective of micropropagation of daylilies to obtain many plants of the same clone, so that flowers will be available in large numbers for the other experiments have been successfully achieved in this research. About 60 plants of cv. Stella D'oro have been successfully transplanted in soil and have since been

producing thousands of flowers each year. In addition to the transplanted plants, there were hundreds of plantlets of all cultivars in tissue culture containers. The other objectives to find out the effect of the position from which the explant was taken on the success of micropropagation of daylily, to use combinations of growth regulators that have not been used in micropropagation of daylily in previous studies and to present the data in a quantitative manner have all been successful.

Optimum conditions for plantlet regeneration were not investigated in this research. However, the findings in this research could be used as a guideline in future experiments with the four cultivars tested towards this objective. Since it is possible to obtain considerably larger numbers of plants using the tissue culture procedure described in this research, this can be used in gene transfer experiments possibly aiming to delay daylily flower senescence using the findings of the other sections of this research.

4.2 DEVELOPMENT AND SENESCENCE OF DAYLILY

4.2.1 General Changes

The experiments on general changes associated with development and senescence of flowers of daylily cultivars were carried out in this research to confirm and extend the findings of previous studies on daylily. The changes in morphology and fresh weight during flower development and senescence of several cultivars that were observed in this study are in agreement with the previous findings on daylily flowers of other cultivars (Lukaszewski and Reid, 1989; Lay-Yee et al., 1992; Bieleski, 1993).

Levels of soluble and total proteins and RNAs also changed as expected. The increase in soluble proteins during flower development and decrease during

senescence has also been observed by Stephenson and Rubeinstein (1998) using the same cv. Stella D'oro. In the cv. Cradle Song, there was a gradual loss of proteins per g fresh weight during bud development and senescence (Lay-Yee et al., 1992). However, it should be noted that their observation was expressed on the basis of fresh weight where as the changes mentioned above on the cv. Stella D'oro were expressed per flower. In agreement with general senescence mechanism of plant parts, SDS PAGE of soluble proteins confirmed losses of certain protein bands and appearance of new protein bands during flower senescence. Loss of protein bands that have also been observed in cv. Cradle Song (Lay-Yee et al., 1992) could be due to the massive hydrolysis of these proteins. Working with flowers of *H. fulva*, Courtney and co-workers (1994) provided further evidence for the degradation of proteins during senescence of daylily flowers. They found that there was an increase in ubiquinated proteins during senescence. The new bands that appear before or during senescence could correspond to the proteins and enzymes that increase during senescence. Several senescence associated-enzymes have been identified in cv. Stella D'oro (Lukaszewski and Reid, 1989; Lay-Yee et al., 1992). In addition there were many genes that are upregulated during senescence of daylily flowers (Guerrero et al., 1998; Valpuesta et al., 1995; Panavas et al., 1998a; 1999).

Insensitivity of daylily flowers to ethylene reported by many previous workers (Lukaszewski and Reid, 1989; Bieleski and Reid, 1993) has also been confirmed in this research. Application of ethylene releasing agent, ethrel or ethylene action inhibitor silver thiosulfate neither delayed nor enhanced senescence of detached flowers of cv. Stella D'oro.

4.2.2 The Problem of Units of Expression

In developmental studies on plants and plant parts, presenting enzyme activities based on weight (fresh or dry) of tissue or total proteins in the extract has serious

drawbacks since both of these parameters also vary during development and senescence. Therefore presenting data based on these two parameters can lead to misunderstanding of the real situation in the system. Because of this, a baseline that is not changing with development should be selected to present enzyme activities. The only parameter that is not changing during development is the structural unit (for example flower, leaf). Thus, in this research, the enzyme activities in most of the experiments have been expressed on the basis of the structural unit (i.e. flower or leaf disc). Of course it is possible to argue that although a flower is an entity that does not change with time, the number of cells per structural unit may change during development of an organ. Trying to express enzyme activities based on the number of cells is an impossible task to carry out in this type of study. Thus, an accurate but practical basis has to be selected to present changes in enzyme activities during development.

4.2.3 Proteolytic Enzymes during Flower Development and Senescence

4.2.3.1 Their Role in Developmental Regulation

During daylily bud development there was little or no detectable levels of endopeptidase activity when gelatin and azoalbumin was used as the substrate. The endopeptidase activity per flower increased after the flower is fully open and reached an optimum 12 h later. This observation is supported by a previous study using gelatin and azocasein as substrates (Guerrero et al., 1998; Stephenson and Rubeinstein, 1998). This is consistent with the previous contention that endopeptidase is responsible for the breakdown of the bulk of proteins in the daylily petals, particularly during the later stages of senescence. The time course study shows that LAP activity seemed to increase before bud opening and reaches a peak 6 h after flower opening. Then it appeared to decrease. This suggests that the LAP in

the daylily petal is also developmentally regulated and is presumably involved in protein turnover during flower growth and could play a special role in the initiation of protein hydrolysis associated with senescence of daylily petals. On the other hand, the carboxypeptidase activity remained unchanged during flower development and senescence. This suggests that this enzyme is not likely to play a special role in protein hydrolysis occurring during daylily petal senescence. This contrasts with that found in tulip petals, where both the carboxypeptidase and LAP activities seemed to increase from flower growth and remained highest during senescence (Sopanan and Carfantan, 1976). By contrast, in potato flowers LAP activity based on the total protein content remained more or less constant during development of bud to open flowers (Herbers et al., 1994). However, this study has not been done on senesced flowers, or on the basis of other parameters. Although the autodigestion of petal proteins during daylily flower senescence has not been studied here, it was found to increase with flower opening in another study using the same cultivar (Stephenson and Rubeinstein, 1998).

To give more credence to the contention that the daylily LAP activity is developmentally regulated i.e. high level of enzyme activity is closely associated with fully-opened and not with senescing daylily flowers, the effect of cycloheximide was investigated. Cycloheximide treatment was previously found to prolong the vase life of cut daylily flowers (Lukaszewski and Reid, 1989), and prevent protein loss associated with flower senescence in cv. Cradle Song (Lay-Yee et al. 1992) and cv. Stella D'oro (Stephenson and Rubinstein 1998). In the latter study, it was shown further that the increase in proteinase activity was prevented by the cycloheximide treatment. In addition, the present study showed that the cycloheximide treatment maintained the high full-bloom levels of the LAP activity and *lap* mRNA as the flower senescence was delayed. Furthermore, several other treatments, for example, 300 mg/L sucrose, were not effective in delaying the senescence of cut daylily flowers and could not prevent the decline in LAP activity associated with flower senescence.

4.2.3.2 Their Role as a Defence Protein

In many plant species, several factors including pathogen attack, mechanical damage and stress conditions are known to produce certain proteins which are thought have a defensive role in protecting the plant. Proteins that are known to appear after pathogen attack are selectively extractable at low pH where they remain soluble while the majority of the other proteins are not (van Loon, 1985; Linthorst, 1991).

In daylily cv. Stella D'oro there was no detectable levels of low pH extractable endopeptidase and LAP in petals, leaves and roots. Application of ABA that induce defence responses in many plant parts (Peña-Cortés et al., 1991; Hildmann et al., 1992) also did not induce synthesis of (neutral pH extractable) LAP in leaf discs and flowers. Similarly, wounding flower parts also did not have any effect on levels of LAP in petals. Low pH extractable carboxypeptidase was also not detected in petals and roots although leaves had detectable levels of the enzyme. It is not possible to assign a defence related role for this carboxypeptidase in daylilies until further studies.

Interestingly, LAP in potato and tomato seems to have a defensive role. In these plants the enzyme is induced in response to wounding, wounding signals, infection by pathogen and insect attack (Bartling and Weiler, 1992; Gu et al., 1996; 1999; Hildmann et al., 1992; Pautot et al., 1993; Bartling and Nosek, 1994; Herbers et al., 1994).

4.2.3.3 Are Proteolytic Enzymes Synthesised or Activated during Daylily Flower Senescence?

Although it is difficult to provide a straightforward answer to the above question with regard to all the three enzymes studied in this research, it is possible to provide some speculation as to what is happening to some of these enzymes in the petals.

The time course study showed that endopeptidase activity starts to increase only after the flower is fully open. Treatment of fully open flowers, which had very low activities of endopeptidase, with cycloheximide delayed senescence possibly by delaying synthesis of endopeptidases that are responsible for the massive hydrolysis of proteins during senescence. Besides, there was no evidence for the presence of an inhibitor or promoter of endopeptidase in daylily petals. Hence, in daylily petals endopeptidase appear to be synthesised rather than activated.

In contrast to endopeptidase, LAP activity was already high when the flowers were treated with cycloheximide. Even if cycloheximide has inhibited further synthesis of LAP in full bloom petals, it does not seem to have imposed a disadvantage on flower life. The mRNA level of *lap* on daylily petals seemed to be higher in buds and in full bloom flowers than in senesced flowers. This suggests that there might be reduced synthesis of LAP during flower senescence. However, the possibility of degradation of LAP during senescence cannot be discarded altogether. The cycloheximide treatment might have delayed the senescence of flowers by delaying synthesis of endopeptidases that are responsible for the breakdown of a lot of proteins including LAP in petals. This could be the reason for the presence of higher levels of LAP in cycloheximide-treated flowers, when compared to water-treated senesced flowers.

Based on the findings of the experiment on endogenous proteinase inhibitors and activators in petals, it is likely that there is no activation or inactivation of the three

enzymes tested in the daylily flower tissues. However, it remains to be seen if in daylily petals there might be proteinaceous inhibitors against proteolytic enzymes of microbial or animal sources. For example, in potato and tomato proteinase inhibitor II (pin2) was constitutively present in flower buds (Peña-Cortés et al., 1991). A proteinase inhibitor that acts against enzyme trypsin was isolated and purified from mature flowers of *Nicotiana plumbaginifolia* (Ausloos et al., 1995). The inhibitor was present in all floral parts of *N. plumbaginifolia* and *N. tabacum*. This inhibitor was thought to have no function in the development of floral parts but to have a protective function against fungal infections and insect damage to floral parts and developing fruits.

4.2.4 LAP in Daylily

Since LAP was developmentally regulated in flowers of daylily cv. Stella D'oro, it would be interesting to know whether this can also be demonstrated in other cultivars of daylily. Of the six cultivars tested, five including cv. Stella D'oro showed a similar pattern of change in the LAP activity. There was an increase in the enzyme activity during bud development and opening and a decrease during senescence. An exception to this pattern was shown by cv. Dallas Star where there was a constant level of LAP in all the three stages of flower development. An explanation for this deviation from the general pattern shown by other five cultivars is beyond the scope of the present research.

Within the daylily cv. Stella D'oro LAP activity was found in leaves, roots, buds, and flowers. In buds and full bloom flowers it was equally distributed in inner and outer petals with little activity in the flower tube. Herbers and co-workers (1994) showed presence of LAP activity in potato leaves and flowers. In the flower it was found in the petals, anthers, ovaries and sepals.

In a previous study where increase in proteinase activity during daylily petal senescence was shown to be accompanied by the appearance of at least 2 new isozymes on protein gels (Stephenson and Rubinstein 1998). To find out if LAP in daylily is also under a similar control mechanism, IEF gel electrophoresis was carried out with daylily tissue extracts. IEF gels showed that there are two isozyme bands of LAP that were run close together in the region with a pI of 5 in leaves, buds and full bloom flowers. In senesced flowers and in roots these bands were very diffuse and almost appeared as one band. In grape berries there were two aminoepeptidase bands with pI values of 4.4. and 5.2 (Pallavicini et al., 1981) while kidney bean cotyledons had a single LAP band with a pI value of 4.8 (Mikkonen, 1992). The tulip petal extracts from fully open and senesced flowers also appear to have two diffuse bands of LAP activities on non-denaturing acrylamide gels although their pIs were not determined (Sopanan and Carfantan, 1976). Although the number of *lap* genes in the daylily or tulip is not known, LAPs in tomato (Pautot et al., 1993) and *A. thaliana* (Bartling and Nosek, 1994) are encoded by small gene families.

4.2.5 Characterisation of LAP

The results from inhibitor study tend to suggest that the LAP in daylily is a metalloenzyme. LAP activity in daylilies was inhibited by EDTA and Zn^{++} but was greatly enhanced by Mn^{++}. In this respect it behaves like alkaline LAPs present in *A. thaliana* (Bartling and Weiler, 1992), tomato (Gu et al., 1999) and potato (Herbers et al., 1994). However, exogenous Mn^{++} was not essential for its activity.

Addition of Mg^{++} had no effect on daylily LAP activity. In addition the enzyme activity was not inhibited by bestatin, an inhibitor specific for certain LAPs, at 0.1 mM concentration that is highly effective on other plant LAPs. Also the enzyme was not stable above 50°C even in the presence of Mn^{++} ions. Therefore the LAP from

daylily appears to be different from thermostable alkaline LAPs of *A. thaliana* (Bartling and Weiler, 1992), tomato (Gu et al., 1999), potato (Herbers et al., 1994) and kidney beans (Mikkonen and Mikola, 1986) which have a high similarity to bovine lens LAP.

NEM a cysteine proteinase inhibitor and a sulfhydryl blocking agent inhibited the LAP activity in daylily petal extracts. In contrast, another cysteine proteinase inhibitor leupeptin did not markedly inhibit LAP activity. On the other hand, a PMSF, serine proteinase inhibitor, was ineffective at 1 mM concentration, a concentration well above the effective concentration against many peptidases. However, at higher concentrations (2 and 3 mM) PMSF inhibited the LAP activity markedly. Furthermore, these high concentrations of PMSF caused precipitation of proteins in the enzyme extracts. Taken all these observations into consideration it is possible to speculate that PMSF could have modified the serine residues in the enzyme molecule which might be critical to the activity of the enzyme.

4.2.6 Purification of LAP Enzyme

Although the attempt to purify the LAP enzyme did not result in a high level of purity in this research, LAPs from other plants have been purified to high levels. A LAP was purified 317 fold with an activity of 9%, from cotyledons of resting kidney beans (*Phaseolus vulgaris* L. cv. Processor) by acidic extraction, ammonium sulphate fractionation and chromatography on DEAE-Sephacel, Sephacryl S-300, Mono Q HPLC and Superose HPLC columns (Mikkonen, 1992). The tomato LAP-A enzyme was overexpressed in *E. coli* and purified to 95% by a process that involves seven steps: IPTG (isopropyl β-D-thiogalactopyranoside) induction, heat denaturation, removal of insoluble proteins, ammonium sulphate precipitation, ion-exchange chromatography and phenyl-superose hydrophobic-interaction

chromatography (Gu et al., 1999). LAP 2 from leaves of *Euonymus alatus* f. ciliato-dentatus was purified by 240-fold (Tazaki and Ishikura, 1984).

During the purification of LAP there was a significant loss of enzyme activity. Activity loss could partly be due to storing the extract between the steps of purification at -20°C. Results showed that LAP activity in daylily was highly susceptible to freezing. In grapes purified aminopeptidase could not be stored at 4°C for 6-8 days without loss of activity in the absence of a protective agent (Pallavicini et al., 1981). Addition of 10% glycerol to the enzyme enabled storage of the enzyme for several months. Similarly, daylily LAP may need some protective agent for its survival during storage. Inability to purify the enzyme while retaining the activity could also be due to lack of protecting agents in the enzyme extract.

4.2.7 Senescence Mechanism

4.2.7.1 Flower Senescence

Various chemicals are known to affect the longevity of detached flowers. The chemicals that prolong flower longevity usually delay or counteract one or more of the changes associated with flower senescence, while those that enhance senescence (e.g. ABA) quicken changes associated with the process by acting together with other associated factors. When detached flowers of daylily cv. Stella D'oro were treated with a variety of chemicals that are known to affect vase life of other flowers, cycloheximide was the only chemical that had an effect. Cycloheximide (at 1 mg/mL) delayed senescence of cut daylily flowers by 2 days. A similar observation, on the detached flowers of the same cultivar, was reported by Stephenson and Rubinstein (1998). In the daylily cv. Cradle Song, cycloheximide abolished the small respiratory climacteric and delayed senescence of detached flowers, petals and petal slices (Bieleski and Reid, 1992). In addition,

cycloheximide has been shown to delay senescence of a variety of flowers including ethylene-sensitive and ethylene-insensitive flowers (Wulster et al., 1982; Jones et al., 1994; Eason and De Vré, 1995; van Doorn et al., 1995; Burge et al, 1996).

Since daylily flower has such a short life span, any chemical that can affect its longevity should have a very strong influence on the senescence process. Cycloheximide, a potent inhibitor of protein synthesis, might have been interfering with multiple senescence associated events that are taking place at a rapid rate in daylily flowers. It might be inhibiting synthesis of a number of senescence-associated proteins and enzymes including synthesis of proteolytic enzymes and thereby delaying senescence. From the results obtained, it is possible that cycloheximide would prevent the breakdown of LAP and other proteins that are associated with petals of full bloom flowers of daylily. Cycloheximide is also known to have some other less known effects on plant tissues. These include increasing respiration, and inhibiting ion uptake in certain storage and leaf tissues (Ellis and MacDonald, 1970), closing stomata and decreasing transpiration and water uptake in cut *Iris* flowers (Mor et al., 1980). In *Leptospermum scoparium* Forst. flowers, cycloheximide extended vase life by 2 days (Burge et al., 1996) by delaying decline in water uptake and leaf moisture content. In this study, cycloheximide is thought to delay induction of stem occlusions of physiological origin. To what extent these factors contribute to the cycloheximide effect on daylily flowers is not known although uncoupling or inhibition of respiration by cycloheximide can be excluded (Lay-Yee et al., 1992) from its effects on daylily flowers.

In daylilies cycloheximide also inhibited bud opening and expansion of detached petals from buds. This observation is in agreement with the previous studies on cv. Stella D'oro (Stephenson and Rubeinstein, 1998) and cv. Cradle Song (Bieleski and Reid, 1993). Inhibition of bud opening could also be attributed to its action as an

inhibitor of protein synthesis. Similar to daylily, application of cycloheximide had prevented bud opening in *Iris* flowers (van Doorn et al., 1995).

In contrast to cycloheximide, chemicals such as antioxidants, amino acids, sugars, antimicrobial compounds and growth regulators that are capable of interfering with one to few senescence processes were not effective enough to prolong the longevity of daylily flowers.

Panavas and Rubinstein (1998) showed that there is a reduction in the level of ascorbic acid during senescence of daylily petals of the same cv. Stella D'oro. In addition there were reductions in levels of ascorbate peroxidase and catalase enzyme that remove hydrogen peroxide while there were increases in levels of hydrogen peroxide and hydrogenase activity. However, exogenous application of ascorbic acid was not effective in delaying senescence of daylily flowers in this study.

In certain detached flowers senescence was related to reduced uptake of water due to formation of vascular occlusions (van Doorn, 1989; van Doorn et al., 1989). Since petals from buds and full bloom flowers treated with these chemicals behaved in the same way as detached flowers this might not be the case in daylilies. Besides, treatment of flowers with 8-hydroxyquinoline, which is known to inhibit bacterial growth in vase solutions which, may result in a blockage of vascular tissues and inhibition of water uptake, had no effect on flower longevity of daylily flowers. 8-hydroxyquinoline enhanced the vase life of ethylene-insensitive flowers of *Gloriosa rothschildiana* by improving water uptake (Jones and Truett, 1992) although it had no effect on vase life of ethylene-insensitive *Sandersonia* flowers (Eason and Webster, 1995). Since uptake of cycloheximide was confirmed by its effect in extending vase life of daylily flowers, vascular blockage due to air embolism could also be discarded in the case of daylily flowers. However, formation of occulations that exclude most other substances cannot be discarded altogether.

During senescence of daylily flowers of cv. Cradle Song glucose and fructose levels increased to a maximum when flowers are fully open and decreased with senescence while sucrose levels remained constant (Bielesky, 1993). But supplying these sugars externally to full bloom flowers did not delay senescence of flowers of cv. Stella D'oro. In contrast to senescence of daylily flowers, senescence of flowers of *Sandersonia aurantiaca* was delayed by sucrose treatment (Eason et al., 1997; 2000a). The difference may lie on the normal life span of the two flowers. *Sandersonia* flowers last for 11 days (Eason and Webster, 1995). Factors that can counteract one to few senescence-associated changes may have an effect on slowing down the senescence process in *Sandersonia* flowers. Similar to *Sandersonia*, ethylene-insensitive flowers of *Gloriosa* (Jones and Truett, 1992) and *Gladiolus* (Bravdo et al., 1974) that have relatively long life spans of 8 and 3-5 (Serek et al., 1994) days respectively had prolonged vase lives in the presence of sucrose.

Although ethylene-insensitive flowers including daylily is not responsive to silver thiosulfate, ethylene-sensitive flowers like *Leptospermum scoparium* had slightly extended vase lives by silver thiosulfate which was achieved not by inhibiting ethylene action but by improving water uptake (Burge et al., 1996). It also improved floret opening in *Gladiolus* flowers (Serek et al., 1994).

Contrary to the lack of effect of glyphosate on daylily flowers, it had enhanced vase life of *Sandersonia* flowers (Eason et al., 2000a), even though senescence of both flowers is insensitive to ethylene. Treatment of *Sandersonia* flowers with phenylalanine and tyrosine removed the beneficial effects of glyphosate.

Application of GA_3 did not affect the flower senescence in daylily cv. Stella D'oro. Similarly, exogenous GA_3 had no effect on the rate of senescence of daylily flowers of cv. Cradle Song (Guerrero et al., 1998). But application of GA_3 to opening buds

of the same cultivar caused an increase in two thiloprotease gene transcripts which are associated with petal senescence (Guerrero et al., 1998).

Cytokinins which are generally known to delay senescence of many flowers (Halevy and Mayak, 1981; Lukaszewska et al., 1994) were not effective in delaying senescence of daylily flowers. Lack of effectiveness of BA in delaying senescence of daylily flowers had also been observed by Lukaszewski and Reid (1989). Similar to BA, kinetin also had no effect on daylily flower senescence.

Although ABA did not enhance senescence of daylily flowers to visually detectable levels in this study, it had been found to prematurely upregulate certain senescence associated events in the same cultivar in a previous study (Panavas et al., 1998b).

This experiment on longevity of flowers was carried out using certain selected chemicals. Although most of these chemicals except cycloheximide were not effective in changing flower longevity, there could be other chemicals, for example growth regulators other than those used here and RNA synthesis inhibitors, that could affect flower life.

Generally plant parts are responsive to chemicals within a certain range of concentrations. While concentrations below this range may not be effective in bringing out the changes, high concentrations may cause toxicity effects. The right concentration to get a response could be found out only by studying the effect of different concentrations of a particular chemical. The concentration of each chemical in this study had been selected by consulting previous literature on similar studies and also by carrying out the study myself to avoid toxic concentrations. However, there is still the possibility that a concentration tested here may not be in the physiologically responsive range.

As mentioned in the introduction, natural senescence is controlled by an overall effect of various factors. Studying the effect of one chemical at a time may provide an idea about the effect of that chemical on longevity. Effect of mixtures of the chemicals had not been studied in this research.

4.2.7.2 Flower Senescence vs. Leaf Yellowing

When compared to petals, daylily leaves appear to behave in a different way. In contrast to flowers whose senescence is accompanied by a reduction in LAP activity, there was no change in LAP activity during yellowing of daylily leaves. This was observed in naturally yellowing intact leaves as well as in dark-induced yellowing leaf discs. This contrasts with the findings of other studies on other plants. In *Euonymus* and potato leaves, there was an increase in LAP activity (Tazaki and Ishikura, 1983; Herbers et al., 1994) whereas in corn and barley, there was a decrease in LAP activity during yellowing of leaves (Feller et al., 1977; Thayer et al., 1988). Similar to LAP in leaves, there was no reduction in soluble protein contents during yellowing of daylily leaves indicating that there was no significant reduction of proteins. This observation is in contrast with the general pattern of massive proteolysis during leaf senescence.

Unlike flowers, daylily leaf discs responded to several chemicals in a manner different to those in water controls. Since leaf yellowing and senescence is a slower process when compared to daylily flower senescence, chemicals that can interfere with one to few senescence associated changes may be able to affect longevity of leaves in a visually detectable level.

By exposing a large number of plants to ethylene Woltering (1987) showed that similar to flowers, leaves of different species also respond differently to ethylene. However, sensitivity of daylily leaves to ethylene has not been tested in previous

studies. Although, flowers are insensitive to ethylene and are considered as modified leaves, it cannot be assumed that leaves are also insensitive to ethylene. At the concentrations tested, ethrel did not cause yellowing of leaf discs at all. This could be due to insensitivity of leaf discs to ethrel exposure, to low levels of ethylene released by the ethrel concentrations tested which were not high enough to cause yellowing of leaf discs or to the level of maturity that makes the leaf discs insensitive to ethylene (Matoo and Aharoni, 1988; Warman and Solomos, 1988). Although ethrel treatment did not cause yellowing of daylily leaf discs, ethylene was effective in yellowing detached *Ivy* leaves (Warman and Solomos, 1988). Ethrel treatment that retained the green colour in leaf discs had very low LAP activity when compared to yellowed discs in water. On the other hand, silver thiosulfate treatment that caused yellowing of leaf discs at the same time as those incubated in water had levels of LAP similar to levels in water-treated yellowed leaf discs.

The observation that ethrel treated leaf discs were limp and delicate whereas water-treated discs were still stiff and turgid should be investigated further. This may be related to the effect of ethylene on cell wall compositions and membranes causing changes in permeability which was observed in ethylene-sensitive flowers (Suttle and Kende, 1978).

Similar to ethrel, cycloheximide which was known to delay yellowing of leaves in other plants (Martin and Thimann, 1972) also retained the green colour of leaf discs and had very low levels of LAP when compared to yellowed leaf discs kept in water. This could be due to the inhibition of protein synthesis which might have prevented synthesis of LAP too.

Ascorbic acid and casein hydrolysate that are known to counteract effects of free radicals and provide amino acids respectively, had no effect in delaying yellowing of daylily leaf discs when compared to water controls. On the other hand, sucrose and fructose had a significant effect in keeping the leaf discs green longer than water

controls. Sugars may be acting as carbon sources under the dark incubation. Leaf discs kept in glucose solution conatining 8-hydroxyquinoline turned brownish around the same time as those kept in 8-hydroxyquinoline alone. Therefore, this browning effect is more likely to be due to the effect of 8-hydroxyquinoline rather than effect of glucose. In oat leaf segments glucose treatment helped to retain higher levels of chlorophyll (Tetley and Thimann, 1974).

Cytokinins and GA_3 are known to retard senescence of intact leaves and leaf discs in certain plants (Fletcher and Osborne, 1966; Beevers, 1966; Goldwaite and Laetsch, 1968; Fletcher, 1969; Tetley and Thimann, 1974; Jordi et al., 1995; Franco and Han 1997) although they are not effective on leaves of some other plants (Goldwaite and Laetsch, 1967). In contrast to its ineffectiveness in delaying daylily flower senescence, BA retained the green colour in leaf discs until the end of the experimental period. BA had shown to be effective in retarding leaf yellowing in *Solidago Canadensis* and Easter lily (Philosphhadas et al., 1996; Franco and Han, 1997). Kinetin that did not affect flower senescence was also not effective in retaining the green colour of leaf discs. Therefore, cytokinins BA and kinetin appear to produce opposite responses as far as yellowing of daylily leaf discs are concerned. However, kinetin had been effective in delaying senescence and yellowing of leaves of other plants (Atkin and Srivastava, 1970; Martin and Thimann, 1972). GA_3 was also slightly better in delaying the yellowing of daylily leaf discs when compared to water controls. On the other hand leaf discs incubated in IAA did not show a response that is different to water control in daylily leaf discs. This observation is in agreement with the observation made on bean leaves where application of IAA had no effect on yellowing of leaves (Goldwaite and Laetsch, 1967).

In contrast to its effects on daylily flowers, ABA accelerated yellowing of leaf discs. This is in agreement with the observation where ABA increased the rate of decline in chlorophyll in radish leaf discs 2 days after incubation (Colquhoun and Hillman, 1972). Contrary to the observation made on yellowed leaf discs kept in water, silver

thiosulfate, and also in yellowed intact leaves, those from the ABA treatment had very low levels of LAP activity. Besides, this reduction in LAP activity when compared to those kept in water controls had occurred within a very short period of 2 days.

4.2.8 Conclusions on the Studies of Proteolytic Enzymes in Daylily Flowers and Leaf Discs

LAP appears to play a developmental role in daylily flowers of at least five cultivars. The enzyme may be responsible at least in part for the initiation of proteolysis in senescing flowers. Endopeptidase is responsible for the hydrolysis of a majority of proteins while carboxypeptidase does not appear to play a special role during daylily flower sensecence. During daylily flower senescence endopeptidase appears to be synthesised rather than activated while there was no evidence for endogenous LAP inhibitors in daylily flower extracts at various times during flower development and senescence.

In daylily cv. Stella D'oro, LAP activity is present in flowers, leaves and roots. There are two isozymes of LAP which run closely or diffused into one band in IEF gels with a pI value of 5.

LAP in daylily seems to be an alkaline metalloenzyme and thermolabile. It is not inhibited by bestatin and appears to be different from thermostable alkaline LAPs found in certain plants and mammalian tissues. It is inhibited by a cysteine proteinase inhibitor NEM and a serine proteinase inhibitor PMSF. Its activity is enhanced by Mn^{++}, inhibited by EDTA and Zn^{++} but not affected by Mg^{++} and leupeptin.

The degradation processes that are taking place in daylily flowers are so intense and rapid, that only cycloheximide that is capable of interfering with many senescence processes can prolong longevity of daylily flowers. On the other hand, daylily leaf yellowing is a slow process. It can be affected by several chemicals that are known to affect flower and leaf senescence of other plants.

In contrast to flowers, daylily leaf yellowing is not accompanied by a reduction in LAP activity. Cycloheximide treatment that retained high LAP activity in flowers and green colour in leaf discs caused significant reductions in LAP activity in leaves. Ethrel treatment also caused reductions in LAP activity. Therefore, cycloheximide and ethrel appear to act in a similar way in retaining green colour of the leaf discs while causing reductions in LAP. Silver thiosulfate that caused yellowing of leaf discs retained levels of LAP similar to yellowed leaf discs kept in water. With regard to this aspect, leaf discs in silver thiosulfate appear to respond in a manner similar to those kept in water. ABA that enhanced yellowing also resulted in reduced levels of LAP. Hence, ABA appears to be reducing LAP in a mechanism different to that of ethrel and cycloheximide.

4.2.9 Recommendations for Future Study on Development and Senescence of Daylily Flowers

The possibility of proteolytic enzymes acting as defence proteins in daylilies had not been investigated in detail in this research. Results of the preliminary studies on this aspect did not provide evidence for a defensive role for these enzymes in daylily tissues, with possible exception for the carboxypeptidase in leaves. It would be interesting to carry out a thorough investigation on these three enzymes in all parts of daylily including fruits and seeds.

Although apparently there were no activators and inhibitors of proteolytic enzymes that act on the substrates used in this study, further studies are required to find out if there are any activators and inhibitors in daylily petal extracts of proteolytic enzymes of microbial or animal sources.

The attempts to purify the LAP from daylily also did not result in the required levels of purification. Therefore alternative purification techniques are needed. In this research, no agents have been added to protect the enzyme during purification. In the future enzyme purification schemes, such measures should be taken into consideration.

Full characterisation of LAP in daylily has a long way to go. Some aspects of characterisation that could be recommended include the determination of the specificity of daylily LAP to different substrates, comparison of daylily LAP to those from other plants via amino acid sequencing.

This study demonstrated that the heterologous *lap* cDNA probe from *Arabidopsis thaliana* could be useful for the study on *lap* gene regulation in daylily. The same probe could be used in Southern blotting to determine the number of *lap* genes in the daylily genome, or screening daylily cDNA or genomic DNA libraries to isolate daylily *lap* gene sequences.

Recently new daylily hybrids with flowers that last for 2 days have been developed. It would be interesting to find out whether the findings of this study, especially the way LAP is regulated in flowers, apply to these hybrids as well. Since LAP appears to be associated with fully open flowers of daylily, it would be fascinating to produce transgenic plants (*lap* mutants as well as plants that overexpress LAP activity) and carry out a similar investigation to further confirm the findings of this research.

Most of the experiments in this thesis had been carried out on daylily petals. But in reality flower senescence is a complex phenomenon where all the floral parts may be interacting with each other to bring about the changes that are taking place during senescence. Studying the contribution of each floral part towards overall development and senescence will be necessary to get a proper understanding of the daylily flower senescence. Studying the petal senescence is just the first step of such a study.

Since LAP in daylily appears to behave differently during leaf and flower senescence, they may be located in different compartments in the cell. Therefore, it would be interesting to determine the location of LAP in daylily flowers and leaves. This might lead to some understanding about the role of the enzyme in the two tissues.

In this research, the effects of various chemicals on yellowing of leaf discs and senescence of daylily flowers were compared. An in-depth study on leaf senescence of other plants having ethylene-insensitive flowers could be very interesting as shown here.

Sensitivity of daylily leaves to ethylene and the effect of ethrel treatment in retaining green colour and softening the tissues of daylily leaf discs also warrant further study.

REFERENCES

Andrews, P. C., Schndeider, F. A., Lebrynk, D. and Butler, L. G., (1979), Maize leaf proteases and their effect on endogenous inorganic pyrophosphate, Biochemistry, 18: 1927-1932.

Anthony, D., (1996), Horticulture's best kept secret, New Zealand Gardner, 52(6): 44-47.

Apps, D. A and Heuser, C. W., (1975), Vegetative propagation of *Hemerocallis*-including tissue culture, The International Plant Propagation Society, 25: 362-367.

Ashman T-L. and Schoen, D. J., (1994), How long should flowers live?, Nature, 371: 788-790.

Atkin, R. K. and Srivastava, B. I. S., (1970), Studies on protein synthesis by scenescing and kinetin-treated barley leaves, Physiol. Plant., 23: 304-315.

Ausloos, J., Proost, P., van Damme, J. and Vendrig, J. C., (1995), Proteinase inhibitor II is developmentally regulated in *Nicotiana* flowers, Physiol. Plant., 94: 701-707.

Back, A. and Richmond, A. E., (1971), Interrelations between gibberellic acid, cytokinins and abscisic acid in retarding leaf senescence, Physiol. Plant., 24: 76-79.

Baes, P. and van Cutsem P., (1992), Chicory seed lot variety identification by leucine-aminopeptidase and esterase zymogram analysis, Electrophoresis, 13: 885-886.

Barret, A. J., (1994), Classification of peptidases, In Methods of enzymology, (Ed.), Barret, A. J., Academic Press, San Diego, 244: 1-18.

Bartling, D. and Nosek, J., (1994), Molecular and immunological characterization of leucine aminopeptidase in *Arabidopsis thaliana*: a new antibody suggests a semi-constitutive regulation of a polygenetically old enzyme, Plant Sci., 9: 199-209.

Bartling, D. and Weiler, E. W., (1992), Leucine aminopeptidase from *Arabidopsis thaliana*, Molecular evidence for a phylogenetically conserved enzyme of protein turnover in higher plants, Eur. J. Biochem., 205: 425-431.

Beevers, L., (1966), Effect of gibberellic acid on the senescence of leaf discs of Nasturtium (*Tropaeolum majus*), Plant Physiol., 41: 1074-1076.

Bieleski, R. L., (1993), Fructan hydrolysis drives petal expansion in the ephemeral daylily flower, Plant Physiol., 103: 213-219.

Bieleski, R. L., (1995), Onset of phloem export from scenescent petals of daylily, Plant Physiol., 109: 557-565.

Bieleski, R. L. and Reid, M. S., (1992), Physiological changes accompanying scenescence in the ephemeral daylily flower, Plant Physiol., 98: 1042-1049.

Biggs, M. S., Harriman, R. W. and Handa, A. K., (1986), Changes in gene expression during tomato fruit ripening, Plant Physiol., 81: 395-403.

Birk, Y., (1976), Proteinase inhibitors from plant sources, In Lorand, L., (Ed.), Methods in enzymology, proteolytic enzymes, Part B, XLV: 695-697.

Bollag, D. M., Rozycki, M. D. and Edelstein, S. J., (1996), Protein methods, Wiley-Liss, NY.

Borochov, A., Halevy, A. H., Borochov, H. and Shinitzky, M., (1978), Microviscocity of plasmalemmas in rose petals as affected by age and environmental factors, Plant Physiol., 61: 812-815.

Borochov, A., Halevy, A. H. and Shinitzky, M., (1976), Increase in microviscocity with aging in protoplast plasmalemma of rose petals, Nature, 263:158-159.

Borochov, A., Halevy, A. H. and Shinitzky, M., (1982), Scenescence and the fluidity of rose petal membranes, relationship to phospholipid metabolism, Plant Physiol., 69: 296-299.

Bradford, M. M., (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein-dye binding, Anal. Biochem., 72: 248-254.

Brady, C. J., Meldrum, S. K., McGlasson, W. B. and Ali, Z. M., (1983), Differential accumulation of the molecular forms of polygalacturonase in tomato mutants, J. Food Biochem., 7: 7-14.

Bravdo, B., Mayak, S. and Gravrieli, Y., (1974), Sucrose and water uptake from concentrated sucrose solutions by *Gladiolus* shoots and the effects of these treatments on floret life, Can. J. Bot., 52: 1271-1281.

Burge, G. K., Bicknell, R. A. and Dobson, B. G., (1996), Postharvest treatment to increase water uptake and the vase life of *Leptospermum scoparium* Forst., New Zealand Journal of Crop and Horticultural Science, 24: 371- 378.

Carrasco, P. and Carbonell, J., (1990), Changes in the level of peptidase activities in pea ovaries during senescence and fruit set induced by gibberellic acid, Plant Physiol., 92: 1070-1074.

Chang, D-Y, Miksche, J. P. and Dhillon, S. S., (1985), DNA changes involving repeated sequences in senescing soybean (*Glycine max*) cotyledon nuclei, Physiol. Plant., 64: 409-417.

Chen, C. H. and Holden, D. J., (1972), Organogenesis in daylily callus, Proc. S. D. Acad. Sci., 51: 146-149.

Chrispeels, M. J. and Boulter, D., (1975), Control of storage protein metabolism in the cotyledons of mung beans: role of endopeptidase, Plant Physiol., 55: 1031-1037.

Christoffersen, R. E., Warm, E. and Laties, G. G., (1982), Gene expression during fruit ripening in avocado, Planta, 155: 52-57.

Chu, I. Y. E. and Kurtz, S. L., (1990), Commercialization of plant micropropagation, In Ammirato, P. V., Evans, D. R., Sharp, W. R. and Bajaj, Y. P. S., (Eds.), Handbook of plant cell culture: ornamental Species, McGraw-Hill Pub. Comp. NY, pp 126-164.

Collier, D. E., (1997), Changes in respiration, protein and carbohydrates of tulip tepals and *Alstroemeria* petals during development, J. Plant Physiol., 150: 446-451.

Colquhoun, A. J., and Hillman, J. R., (1972), The effect of abscisic acid on senescence in leaf discs of radish, *Raphanus sativus* L., Planta (Berl.), 105: 213-224.

Courtney, S. E., Rider, C. C. and Stead, A. D., (1994), Changes in protein ubiquitination and the expression of ubiquitin-encoding transcripts in daylily petals during floral development and scenescence, Physiol. Plant., 91: 196-204.

Coux, O. and Piechaczyk, M., (2000), The ubiquitin/proteasome pathway, M-S-medicine Science, 16(5): 623-629.

Degan, F. D., Rocher, A., Cameron-Mills, V. and von Wettstein, D., (1994), The expression of serine carboxypeptidases during maturation and germination of the barley grain, Proc. Natl. Acad. Sci. USA, 91: 8209-8213.

DellaPenna, D., Kates, D. S. and Bennett, A. B., (1987), Polygalacturonase gene expression in Rutgers, *rin*, *nor*, and *Nr* tomato fruits, Plant Physiol., 85: 502-507.

Deurenberg, J. J. M., (1976), Activation of protein synthesis in ovaries from *Petunia hybrida* after compatible and incompatible pollination, Acta Bot. Neerl., 25(3): 221-226.

Deurenberg, J. J. M., (1977a), Differentiated protein synthesis with polysomes from *Petunia* ovaries before fertilization, Planta, 133: 201-206.

Deurenberg, J. J. M., (1977b), Evidence against the formation of fast diffusing substances preceding fertilizaion in petunia, Acta Bot. Neerl., 26(4): 353-355.

Dodds, J. H. and Roberts, L. W., (1985), Experiments in plant tissue culture, Cambridge University Press, Cambridge.

Doi, E., Komori, N., Matoba, T. and Morita, Y., (1980), Some properties of carboxypeptidases in germinating rice seeds and rice leaves, Agric. Biol. Chem., 44(1): 77-83.

Droillard, M-J. and Pauline, A., (1990), Isozymes of superoxide dismutase in mitochondria and peroxisomes isolated from petals of carnation (*Dianthus caryophyllus*) during senescence, Plant Physiol., 94: 1187-1192.

Eason, J. R. and De Vré, L., (1995), Ethylene-insensitive floral scenescence in *Sandersonia aurantiaca* (Hook.), New Zealand Journal of Crop and Horticultural Science, 23: 447-454.

Eason, J. R., De Vré, L. A., Somerfield, S. D. and Heyes, J. A., (1997), Physiological changes associated with *Sandersonia aurantiaca* flower senescence in response to sugar, Postharvest Biol. Technol., 12: 43-50.

Eason, J. R., Johnston, J. W. and De Vré, L., (2000a), Reversal of glyphosate inhibition of *Sandersonia aurantiaca* flower senescence with aromatic amino acids, Postharvest Biol. Technol., 18: 81-84.

Eason, J. R., Johnston, J. W., De Vré, L., Sinclair, B. K. and King, G. A., (2000b), Amino acid metabolism in senescing *Sandersonia aurantiaca* flowers: cloning and characterization of asparagine synthatase and glutamine synthatase cDNAs, Aust. J. Plant Physiol., 27: 389-396.

Eason, J. R. and Webster, D., (1995), Development and scenescence of *Sandersonia aurantiaca* (Hook.) flowers, Sci. Hortic., 63: 113-121.

Elleman, T. C., (1974), Aminopeptidase of pea, Biochem. J., 141: 113-118.

Ellis, R. J. and MacDonald, I. R., (1970), Specificity of cycloheximide in higher plant systems, Plant Physiol., 46: 227 –232.

Erhardt, W., (1992), *Hemerocallis* daylilies, D. Grenfell, (Ed.), Translation A. Helm, Kangaroo Press, NSW.

Even-Chen, Z., Atsmon, D. and Itai, C., (1978), Hormonal aspects of senescence in detached tobacco leaves, Physiol. Plant., 44: 377-382.

Even-Chen, Z. and Itai, C., (1975), The role of abscisic acid in senescence of detached tobacco leaves, Physiol. Plant., 34: 97-100.

Feller, U. K., Soong, T. T. and Hageman, R. H., (1977), Leaf proteolytic activities and senescence during grain development of field grown corn (*Zea mays* L.), Plant Physiol., 59: 290-294.

Ferrasson, E., Quillien, L. and Gueguen, J., (1997), Proteinase inhibitors from pea seeds: purification and characterization, Journal of Agricultural and Food Chemistry, 45(1): 127-131.

Fitter, M. S. and Krikorian, A. D., (1981), Recovery of totipotent cells and plantlet production from daylily protoplasts, Ann. Bot., 48: 591-597.

Fletcher, R. A., (1969), Retardation of leaf senescence by benzyladenine in intact bean plants, Planta, 89: 1-8.

Fletcher, R. A., and Osborne, D. J., (1966), Gibberllin, as a regulator of protein and ribonucleic acid synthesis during senescence in leaf cells of *Taraxacum officinale*, Can. J. Bot., 44: 739-745.

Franco, R. E. and Han, S. S., (1997), Respiratory changes associated with growth-regulator-delayed leaf yellowing in Easter lily, J. Am. Soc. Hortic. Sci., 122(1): 117-121.

Garello, G., Ménard, C. and Dansereau, B., (1995), The influence of light quality on rose flower senescence; involvement of abscisic acid, Plant Growth Reg., 16: 135-139.

Gear, I., (1998), Daylilies in New Zealand, In D. Grenfell, (Ed.), The gardener's guide to growing daylilies, pp 141-143.

Georgakopoulos, J-H. and Argyroudi, A-J-H., (1997), Implication of D1 degradation in phosphorylation-induced state transitions, Photosynthesis-research, 53(2-3), 185-195.

Gepstein, S., (1998), Photosynthesis, In Noodén, L. D. and Leopold, A. C., (Eds.), Senescence and ageing in plants, Academic Press Inc., San Diego, pp 85-104.

Gilissen, L. J. W., (1976), The role of the style as a sense-organ in relation to wilting of the flower, Planta, 131: 201-202.

Gilissen, L. J. W. and Hoekstra, F. A., (1984), Pollination-induced corolla wilting in *Petunia hybrida* rapid transfer through the style of a wilting-inducing substance, Plant Physiol., 75: 496-498.

Goeden-Kallemeyn, Y. C. and Chen C. H., (1978), Consistency of the chromosome constitition in plants derived from a long term daylily callus culture, Proc. S. D. Acad. Sci., 57: 108-112.

Goldthwaite, J. J. and Laetsch, W. M., (1967), Regulation of senescence in bean leaf discs by light and chemical growth regulators, Plant Physiol., 42: 1757-1762.

Goldthwaite, J. J. and Laetsch, W. M., (1968), Control of senescence in *Rumex* leaf discs by gibberellic acid, Plant Physiol., 43: 1855-1858.

Green, T. R. and Ryan, C. A., (1972), Wound-induced proteinase inhibitor in plant leaves: a possible defence mechanism against insects, Science, 175: 776-777.

Grenfell, D., (1998), The gardner's guide to growing daylilies, Timber Press, Portland, Orgon.

Gu, Y-Q., Holzer, F. M. and Walling, L. L., (1999), Overexpression, purification and biochemical characterization of the wound-induced leucine aminopeptidase of tomato, E. J. Biochem., 263: 726-735.

Gu, Y-Q., Pautot, V., Holzer, F. M. and Walling L. L., (1996), A complex array of proteins related to the multimeric leucine aminopeptidase of tomato, Plant Physiol., 110: 1257-1266.

Guerrero, C., Calle, M. de la, Reid, M. S. and Valpuesta, V., (1998), Analysis of the expression of two thiloprotease genes from daylily (*Hemerocallis* spp.) during flower senescence, Plant Mol. Biol., 36: 565-571.

Halaba, J. and Rudnicki, R. M., (1983), An invertase inhibitor as affecting wilting of carnation petals, Acta. Hortc., 138: 261-267.

Hamato, N., Koshiba, T., Pham, T. N., Tatsumi, Y., Nakamura, D., Takano, R., Hayashi, K., Hong, Y. M. and Hara, S., (1995), Trypsin and elastate inhibitors from bitter gourd (*Momordica charantia* Linn.) seeds, J. Biochem. (Tokyo), 117(2): 432-437.

Halevy, A. H. and Mayak, S., (1979), Scenescence and postharvest physiology of cut flowers-Part 1, In Janick, J., (Ed.), Horticultural Reviews, Vol. 1, Avi Publishing, Westport, Connecticut, pp 204-236.

Halevy, A. H. and Mayak, S., (1981), Scenescence and postharvest physiology of cut flowers-Part 2, In Janick, J., (Ed.), Horticultural Reviews, Vol. 3, Avi Publishing, Westport, Connecticut, pp 59-143.

Hass, G. M. and Ryan, C. A., (1981), Carboxypeptidase inhibitor from potatoes, In Lorand, L., (Ed.), Methods in enzymology, proteolytic enzymes, part C, Academic Press Inc., NY., Vol 80, pp 778-791.

Hawker, J. S., Walker, R. R. and Ruffner, H. P., (1976), Invertase and sucrose synthase in flowers, Phytochem., 15: 1441-1443.

Heath, R. L., McDonald, G., Christeller, J. T., Lee, M., Bateman, K., West, J., Van, H. R. and Anderson, M. A., (1997), Proteinase inhibitors from *Nicotiana alata* enhance plant resistance to insect pests, J. Insect Physiol., 43(9): 833-842.

Herbers, K., Prat, S. and Wllimitzer, L., (1994), Functional analysis of a leucine aminopeptidase from *Solanum tuberosum* L., Planta, 194: 230-240.

Heuser, C. W. and Apps, D. A., (1976), *In vitro* plantlet formation from flower petal explants of *Hemerocallis* cv. Chipper Cherry, Can. J. Bot., 54: 616- 618.

Hildmann, T., Ebneth, M., Peña-Cortés, H., Sánchez-Serrano, J. J., Willmitzer, L. and Prta, S., (1992), General roles of abscisic and jasmonic acids in gene activation as a result of mechanical wounding, Plant Cell, 4: 1157-1170.

Ho, L. C. and Nichols, R., (1977), Translocation of ^{14}C-sucrose in relation to changes in carbohydrate content in rose corollas cut at different stages of development, Ann. Bot., 41: 227-242.

Hoekstra, F. A. and Wegews, R., (1986), Lack of control by early pistillate ethylene of the accelerated wilting of *Petunia hybrida* flowers, Plant Physiol., 80: 403-408.

Hu, S-Y., (1964), Other uses of day-lilies, Garden Journal, 14: 223-228.

Hussey, G., (1983), *In vitro* propagation of horticultural and agricultural crops, In Mantell S. H. and Smith, H., (Eds.), Plant Biotechnology, Cambridge University Press, Cambridge, pp-111-138.

International Union of Biochemistry and Molecular Biology, (1992), Enzyme nomenclature, Academic Press Inc., San Diego.

Isola, M. C. and Franzoni, L., (1996), Aminopeptidase activities in peanut cotyledons, R. Bras. Fisiol. Veg., 8(3): 167-173.

John, I., Drake, R., Farrell, A., Cooper, W., Lee, P., Horton, P. and Grierson, D., (1995), Delayed leaf senescence in ethylene-deficient ACC-oxidase antisense

tomato plants: molecular and physiological analysis, The Plant Journal, 7(3): 483-490.

Jones, O. P., (1983), *In vitro* propagation of tree crops, In Mantell S. H. and Smith, H., (Eds.), Plant Biotechnology, Cambridge University Press, Cambridge, pp-139-159.

Jones, R. B., Serek, M., Kuo, C. and Reid, M. S., (1994), The effect of protein synthesis inhibition on petal scenescence in cut bulb flowers, J. Am. Soc. Hort. Sci., 119(6): 1243-1247.

Jones, R. B. and Truett, J. K., (1992), Postharvest handling of cut *Gloriosa rothschildiana* O'brien (Liliaceae) flowers, J. Am. Soc. Hort. Sci.,117(3): 442-445.

Jordi, W., Stoopen, G. M., Kelepouris, K. and Vanderkrieken, W. M., (1995), Gibberellin-induced delay of leaf senescence of *Alstroemeria* cut flowering stems is not caused by an increase in the endogenous cytokinin content, J. Plant Growth Reg., 14(3): 121-127.

Kaga, M. M., Laurent, F., Doh, A., Luffau, G, Yvoré, P. and Péry, P., (1998), Purification of leucine aminopeptidase from *Eimeria falciformis*, Vet. Res., 29: 107-111.

Kenis, J. D., Silvente, S. T. and Trippi, V. S., (1985), Nitrogen metabolism and scenescence-associated changes during growth of carnation flowers (*Dianthus caryophyllus*), Physiol. Plant., 65: 455-459.

Kenny, A. J., (1999), Introduction: nomenclature and classes of peptidases, In Sterchi, E. E. and Stöcker, W., (Eds.), Proteolytic enzymes, tools and targets, Springer-Verlag,, Berlin, pp 1-10.

Kierszenbaum, A. U., (2000), The 26s proteasome: ubiquitin mediated proteolysis is the tunnel, Molecular reproduction and development, 57(2): 109-110.

Klerk, and van Loon, L-C., (1997), Characterization of protein turnover in the developing first leaf of oats (*Avena sativa* L.) J. Plant Physiol., 151(2): 176-187.

Knight, C. G., (1999), Kinetic analysis of protease inhibition by synthetic inhibitors, In Sterchi, E. E. and Stöcker, W., (Eds.), Proteolytic enzymes, tools and targets, Springer-Verlag, Berlin, pp 167-187.

Kolehmainen L. and Mikola, J., (1971), Partial purification and enzymatic properties of an aminopeptidase from barley, Arch. Biochem. Biophys., 145: 633-642.

Kouzuma, Y., Kawano, K., Kimura. M., Yamasaki, N., Kadowaki, T. and Yamamato, K., (1996), Purification, characterization and sequencing of two cysteine proteinase inhibitors, J. Biochem. (Tokyo), 199(6): 1106-1113.

Krikorian, A. D., Cronauer-Mitra, S. S. and Corbin, M. S. F., (1988), Protoplast culture of perennials, Sci. Hort., 37: 277-293.

Krikorian, A. D. and Kann R. P., (1977), Clonal micropropagation of daylilies, In Sharp, W. R., Larsen, P. O., Paddock, E. F and Raghavan, V., (Eds.), Plant cell and tissue culture, principles and applications, Ohio State University Press, Columbus, pp 835- 836.

Krikorian, A. D. and Kann R. P., (1979), Micropropagation of daylily through aseptic culture techniques, its basis, status, problems and prospects, *Hemerocallis* Journal, 33(1): 44-61.

Krikorian, A. D. and Kann R. P., (1980), Mass blooming of a daylily clone reared from cultured tissue, *Hemerocallis* Journal, 34(1): 35-38.

Krikorian, A. D. and Kann R. P., (1981), Plantlet production from morphogenetically competent cell suspensions of daylily, Ann. Bot., 47: 679-686.

Krikorian, A. D., Kann R. P. and Smith, D. L., (1995), Somatic Embyogenesis in Daylily (*Hemerocallis*), In Bajaj, Y. P. S., (Ed.), Biotechnology in Agriculture and Forestry, Vol 31, Somatic Embryogenesis and Synthetic Seed II, Springer-Verlag, Berlin, pp 285-293.

Krikorian, A. D., O'connor., S. A., Fitter M. S. and Kann, R. P., (1982), Karyotype analysis of daylily plants clonally derived from a long term totipotent suspension culture, In Fujiwara, A., (Ed.), Plant Tissue Culture, The Japanese Association for plant tissue culture, Tokyo, pp 429-430.

Kruger, J. E. and Preston, K. R., (1978), Changes in aminopeptidases of wheat kernels during growth and maturation, Cereal Chem., 55(3): 360-372.

Kuraishi, S., (1968), The effect of kinetin on protein level of *Brassica* leaf disks, Physiol. Plant., 21: 78-83.

Laemmli, U. K., (1970), Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature, 227: 680-685.

Lawton, K. A., Huang, B., Goldsbrough, P. B. and Woodson, W. R., (1989), Molecular cloning and characterization of scenescence-related genes from carnation flower petals, Plant Physiol., 90: 690-696.

Lay-Yee, M., Stead, A. D. and Reid, M. S., (1992), Flower scenescence in daylily (*Hemerocallis*), Physiol. Plant., 86: 308-314.

Leopold, A. C., (1961), Senescence in plant development, Science, 134: 1727-1732.

Leshem, Y. Y., Halevy, A. H. and Frenkel, C., (1986), Processes and control of plant senescence, Elsevier, Amsterdam.

Leung, D. W. M. and Bewley, J. D., (1983), Increased activity of aminopeptidase in the cotyledons of red light-promoted lettuce seeds is controlled by the axis, Physiol. Plant., 59:127-133.

Ling, J-T., and Sauve, R. J., (1995), Isolation and culture of daylily mesophyll protoplasts, Plant Cell Rep., 15: 293-296

Linthorst, H. J. M., (1991), Pathogenesis-related proteins of plants, Critical reviews in plant sciences, 10(2): 123-150

Lovel, P. H., Lovel, P. J. and Nichols, R., (1987), The importance of the stigma in flower senescence in petunia (*Petunia hybrida*), Ann. Bot., 60: 41-47.

Lukaszewska, A. J., Bianco, J., Barthe, P. and Le Page-Degivry, M. T., (1994), Endogenous cytokinins in rose petals and the effect of exogenously applied cytokinins on flower senescence, Plant Growth Reg., 14: 119-126.

Lukaszewski, T. A. and Reid, M. S., (1989), Bulb-type flower scenescence, Acta Hort., 261: 59-62.

Makrides, S. C. and Goldwaite, J., (1981), Biological changes during bean leaf growth, maturity and senescence, content of DNA, polyribosomes, ribosomal RNA, protein and chlorophyll, J. Exp. Bot., 32(129): 725-735.

Malik, N. S. A and Thimann, K. V., (1980), Metabolism of oat leaves during senescence, changes in ATP levels, Plant Physiol., 65: 855-858.

Maniatis, T., Fritsch, E. F. and Sambrook, J., (1982), Molecular cloning, a laboratory manual, Cold Spring Harbor Laboratory, NY.

Martin, C. and Thimann, K. V., (1972), The role of protein synthesis in the senescence of leaves, Plant Physiol., 49: 64-71.

Matile, P. and Winkenbach, F., (1971), Function of lysosomes and lysosomal enzymes in senescing corolla of the morning glory (*Ipomoea purpurea*), J. Exp. Bot., 22(73): 759-771.

Matoba, T. and Doi, E., (1974), Carboxypeptidase activity of tomato fruit during the ripening process and some enzymatic properties, Agr. Biol. Chem., 38(10): 1901-1905.

Mattoo, A. K. and Aharoni, N., (1988), Ethylene and plant senescence, In (Eds.) Noodén, L. D. and Leopold, A. C., Senescence and ageing in plants, Academic Press Inc., San Diego, pp 242-269.

Maxie, E. C., Farnham, D. S., Mitchell, F. G., Sommer, N. F., Parsons, R. A., Snyder, R. G. and Rae, H. L., (1973), Temperature and ethylene effects of cut flowers of carnation (*Dianthus carophyllus* L.), J. Am. Soc. Hort. Sci., 98(6): 568-572.

Mayak, S. and Halevy, A. H., (1980), Flower scenescence, In Thimann, K., (Ed.), Scenescence in plants, CRC Press, Boca Raton, FL, pp 131-156.

McHale, J. S. and Dove, L. D., (1968), Ribonuclease activity in tomato leaves as related to development and senescence, New Phytol., 67: 505-515.

Mckenzie, R. J. and Lovell, P. H., (1992), Flower senescence in monocotyledons: a taxonomic survey, New Zealand Journal of Crop and Horticultural Science, 20: 67-71.

Mehta, R. A., Warmbardt, R. D. and Matoo, A. K., (1996), Tomato fruit carboxypeptidase, properties, induction upon wounding, and immunocytochemical localization, Plant Physiol., 100: 883-892.

Meyer, Jr., M. M., (1976), Propagation of daylilies by tissue culture, Hort Sci., 11(5): 485-487.

Mikkonen, A., (1992), Purification and characteization of leucine aminopeptidase from kidney bean cotyledons, Physiol. Plant., 84: 393-398.

Mikkonen, A, and Mikola , J., (1986), Separation and partial characterization of two alkaline peptidases from cotyledons of resting kidney beans, *Phaseolus vulgaris*, Physiol. Plant., 68: 81-85.

Mikkonen, A, Porali, I, Cercos, M. and Ho, T. H. D., (1996), A major cystein proteinase, EPB, in germinating barley seeds: structure of two intronless genes and regulation of expression, Plant Mol. Biol., 31(2): 239-254.

Mikola, L., (1983), Germinating barley grains contain five acid carboxypeptidases with complementary substrate specificities, Biochim. Biophys. Acta, 747: 241-252.

Mikola, L. and Mikola, J., (1986), Occurrence and properties of different types of peptidases in higher plants, In Dalling, M. J. D., (Ed.), Plant proteolytic enzymes, Vol. 1, CRC Press, Boca Raton, FL, pp 98-117.

Mor, Y., Reid, M. S. and Kofranek, A. M., (1980), Role of the ovary in carnation senescence, Sci. Hort., 13: 377-383.

Mullin, M., (1970), Tissue culture of some monocotyledonous plants, Aust. J. Biol. Sci., 23: 473-477.

Murashige, T. and Skoog, F., (1962), A revised medium for rapid growth and bio-assays with tobacco tissue culture, Physiol. Plant., 15: 473-497.

Natacha-B, Lorette-A, Christel, C., Patrick, G. and Michel, D., (1998), Accumulation and degradation of Em proteins in Arabidopsis thaliana: evidence for post transcriptional controls, J. Expt. Bot., 49(329): 1925-1933.

Nichols, R., (1971), Induction of flower senescence and gynaecium development in the carnation (*Dianthus caryophyllus*) by ethylene and 2-chloroethylphosphonic acid, J. Hort. Sci., 46: 323-332.

Nichols, R., Bufler, G., Mor, Y., Fujino, D. W. and Reid, M. S., (1983), Changes in ethylene production and 1-aminocyclopropane-1-carboxylic acid content of pollinated carnation flowers, J. Plant Growth Reg., 2: 1-8.

Nichols, R. and Ho, L. C., (1975), Effect of ethylene and sucrose on translocation of dry matter and ^{14}C-sucrose in the cut flower of the glasshouse carnation (*Dianthus caryophyllus*) during scenescence, Ann. Bot., 39: 287-296.

Noodén, L. D., (1988a), The phenomena of senescence and ageing, In Noodén, L. D. and Leopold, A. C., (Eds.), Senescence and ageing in plants, Academic Press Inc., San Diego, pp 2-38.

Noodén, L. D., (1988b), Whole plant senescence, In Noodén, L. D. and Leopold, A. C., (Eds.), Senescence and ageing in plants, Academic Press Inc., San Diego, pp 329-427.

Noodén, L. D., (1988c), Abscisic acid, auxin, and other regulators of senescence, In Noodén, L. D. and Leopold, A. C., (Eds.), Senescence and ageing in plants, Academic Press Inc., San Diego, pp 330-355.

Noodén, L. D., Guiamét, J. J. and John, I., (1997), Senescence mechanisms, Physiol. Plant., 101: 746-753.

Norman, A., (1964), Increasing Plants, American *Hemerocallis* Society, 18: 70

Oh, S. A., Lee, S. Y., Chung, I. K., Lee, C-H, Nam, H. G., (1996), A senescence-associated gene of *Arabidopsis thaliana* is distinctively regulated during natural and artificially induced leaf senescence, Plant Mol. Biol., 30: 739-754.

Ostersetzer, O. tabak, S., yarden, O., Sharpira, R. and Adams, S., (1996), Immunological detection of proteins similar to bacterial proteases in a higher plant chloroplast, Eur. J. Biochem., 236(3): 932-936.

Pallavicini, C., Peruffo, A. D. B. and Santoro, M., (1981), Isolation and partial characterization of grape aminopeptidase, J. Agric. Food Chem., 29: 1216-1220.

Panavas, T., Pikula, A., Reid, P. D., Rubinstein, B. and Walker, E. L., (1999), Identification of senescence-associated genes from daylily petals, Plant Mol. Biol., 40: 237- 248.

Panavas, T., Reid, P. D. and Rubinstein, B., (1998a), Programmed cell death of daylily petals: activities of wall-based enzymes and effects of heat shock, Plant Physiol. Biochem., 36(5): 379-388.

Panavas, T. and Rubinstein, B., (1998), Oxidative events during programmed cell death of daylily (*Hemerocallis* hybrid) petals, Plant Sci., 133: 125-138.

Panavas, T., Walker, E. L. and Rubinstein, B., (1998b), Possible involvement of abscisic acid in senescence of daylily petals, J. Exp. Bot., 49(329): 1987-1997.

Pautot, V., Holzer, F. M., Reisch, B. and Walling, L. L., (1993), Leucine aminopeptidase: an inducible component of the defence response in *Lycopersicon esculentum* (tomato), Proc. Natl. Acad. Sci., USA, 90: 9906-9910.

Peña-Cortés, H., Willmitzer, L. and Sánchez-Serrano, J. J., (1991), Abscisic acid mediates wound induction but not developmental-specific expression of the proteinase inhibitor II gene family, Plant Cell, 3: 963-972.

Pfeiffer, H., (1928), *Die Pflanzlichen Trennungsgwebe, Handbuch der Pflanzenanatomie, 1, Abteilung 2, Teil: Histologie. Band V.* Ed. K. Linsbauer, Verlag Gebrüder Borntraeger, Berlin, pp 236.

Philosophhadas, S., Michaeli, R., Reuveni, Y. and Meir, S., (1996), Benzyladenine pulsing retards leaf yellowing and improves quality of goldenrod (*Solidago Canadensis*) cut flowers, Postharvest Biol. And Tech., 9(1): 65-73.

Piskornik, Z., (1986), The role of ethylene in the pollination and senescence of flowers of bulbous plants, Acta Hort., 181: 407-413.

Polgár, L., (1999), Basic kinetic mechanisms of proteolytic enzymes, In Sterchi, E. E. and Stöcker, W., (Eds.), Proteolytic enzymes, tools and targets, Springer-Verlag, Berlin, pp 148-166.

Prescott, J. M. and Wilkes, S. H., (1966), *Aeromonas* aminopeptidase: purification and some general properties, Arch. Biochem. Biophys., 117: 328-336.

Ragster, L. V. and Chrispeels, M. J., (1979), Azocoll-digesting proteinases in soybean leaves, characteristics and changes during leaf maturation and senescence, Plant Physiol., 64: 857-862.

Rappay, G., (1989), Proteinases and their inhibitors in cells and tissues, Progress in histochemistry and cytochemistry, Gustav Fischer Verlag, Stuttgart, NY, Vol 18, No 4.

Reid, M. S. (1989), The role of ethylene in flower scenescence, In Mayak, S. (Ed.), Acta Horticulturae, Forth International Symposium on Postharvest Physiology of Ornamental Plants, No. 261, pp 157-169.

Reid, M. S., Paul, J. L., Farhoomand, M. B. Kofranek, A. M. and Staby, G. L., (1980), Pulse treatments with the silver thiosulfate complex extend the vase life of cut carnations, J. Am. Soc. Hort. Sci., 105(1): 25-27.

Robb, S. M., (1957), The culture of excised tissue from bulb scales of *Lilium speciosum* Thun., J. Exp. Bot., 8(24): 348-352.

Ryan, C. A. and Walker-Simmons, M., (1981), Plant proteinases, In Marcus, A., (Ed.), The biochemistry of plants, Academic Press, New York, 6: 321-350.

Sacher, J. A., (1973), Scenescence and postharvest physiology, Annu. Rev. Plant Physiol., 24: 197-310.

Sagawa, Y. and Kunisaki, J. T., (1990), Micropropagation of floriculture crops, In Ammirato, P. V., Evans, D. R., Sharp, W. R. and Bajaj, Y. P. S., (Eds.), Handbook of plant cell culture: ornamental Species, Vol. 5, McGraw-Hill Pub. Comp. NY, pp 25-56.

Sanderink, G-J, Artur, Y. and Siest, G., (1988), Human aminopeptidases: A review of the literature, J. Clin. Chem. Biochem., 26: 795-807.

Santino, A., Gallo, A., Leone, A., Poltronieri, P. and Zacheo, G., (1998), Purification and characterization of a novel papain inhibitor from common bean (*Phaseolus vulgaris*) seeds, Physiol. Plant., 104(3): 293-298.

Sarath, G., La Motte R. S. de and Wagner, F. W., (1989), Protease assay methods, In Beynon, R. j. and Bond, J. S., (Eds.), Proteolytic enzymes, a practical approach, IRL Press, Oxford, pp 25-55.

Schenk, R. U. and Hildebrandt, A. C., (1972), Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultivars, Can. J. Bot., 50: 199-204.

Scott, G. K. and Symes, C. W. (1996), Isolation, characterisation and cell-growth-regulatory proteins of kumara (sweet potato) trypsin inhibitors, Biochemistry and Molecular Biology International, 38(2): 333-344.

Serek, M., Jones, R. B. and Reid, M. S., (1994), Role of ethylene in opening and scenescence of *Gladiolus* sp. flowers, J. Am. Soc. Hort. Sci., 119(5): 1014-1019.

Shibaoka, H. and Thimann, K. V., (1970), Antogonisms between kinetin and amino acids, Plant Physiol., 46: 212-220.

Sheehy, R. E., Kramer, M. and Hiatt, W. R., (1988), Reduction of polygalacturonase activity in tomato fruit by antisense RNA, Proc. Natl. Acad. Sci. USA, 85; 8805-8809.

Silva, F. F. F., Lima, C. S., Val, R. R. D. and Xavier, F-J., (1995), Cysteine proteinase inhibitors from seeds of *Adenanthera pavonine*, Revista Brasileira de Botanica, 18(2): 137-141.

Slater, A., Maunders, M. J., Edwards, K., Schuch, W. and Grierson, D., (1985), Isolation and characterisation of cDNA clones for tomato polygalacturonase and other ripening-related proteins, Plant Mol. Biol., 5: 137-147.

Smith, D. L. and Krikorian, A. D., (1991), Growth and maintenance of an embryogenic cell culture of daylily (*Hemerocallis*) on hormone-free medium, Ann. of Bot., 76: 443-449.

Smillie, R. M., (1962), Photosynthetic and respiratory activities of growing pea leaves, Plant Physiol., 37: 716-721.

Sopanen, T. and Carfantan, N., (1976), Activities of various peptidases in the senescing petals of tulip, Physiol. Plant., 36: 247-250.

Sopanen, T. and Mikola, J. (1975), Purification and partial characterization of barley leucine aminopeptidase, Plant Physiol., (1975), 55: 809-814.

Spetea, C., Hundal, T., Lohmann, M. and Anderson, B., (1999), GTP bound to chloroplast thylakoid membranes is required for light-induced, multienzyme degradation of the photosystem II D1 protein, Proc. Natl. Aca. Sci. USA, 96(11): 6547-6552.

Stead, A. D. and Moore, K. G., (1977), Flower development and scenescence in *Digitalis purpurea* L., cv. Foxy, Ann. Bot., 41: 283-292.

Stead, A. D. and Moore, K. G., (1979), Studies on flower longevity in *Digitalis*, Planta, 146: 409-414.

Stephenson, P., and Rubinstein, B., (1998), Characterization of proteolytic activity during senescence in daylilies, Physiol. Plant., 104: 463-473.

Stimart, D. P., Brown, D. J. and Solomos, T., (1983), Development of flowers and changes in carbon dioxide, ethylene, and various sugars of cut *Zinnia elegans* Jacq., J. Am. Soc. Hort. Sci., 108(4): 651-655.

Stout, A. B., (1934), Daylilies, MacMillan Co., NY.

Strode, R. E. and Oglesby, R. P., (1976), Commercial tissue culturing at Oglesby nursery Inc., Proc. Fla. State Hort. Soc., 89: 367- 368.

Studier, F. W., (1973), Analysis of bacteriophage T7 early RNAs and proteins on slab gels, J. Mol. Biol., 79: 237-248.

Suttle, J. C. and Kende, H., (1978), Ethylene and scenescence in petals of *Tradescantia*, Plant Physiol., 62: 267-271.

Suttle, J. C. and Kende, H., (1980), Ethylene action and loss of membrane integrity during petal scenescence in *Tradescantia*, Plant Physiol., 65: 1067-1072.

Taylor, A., (1993), Aminopeptidase, structure and function, FASEB J., 290-298.

Tazaki, K. and Ishikura, N., (1983), Multiple forms of aminopeptidase in *Euonymus* leaves, Plant and Cell Physiol., 24: 1263-1268.

Tazaki, K. and Ishikura, N., (1984), Purification and characterization of an aminopeptidase, LPAase 2, from *Euonymus* leaves, Plant and Cell Physiol., 25(5): 731-737.

Tetley, R. M. and Thimann, K. V., (1974), The metabolism of oat leaves during senescence, 1. respiration, carbohydrate metabolism, and the action of cytokinins, Plant Physiol., 54: 294-303.

Thaler, J. S., Stout, M. J., Karban, R. and Duffey, S. S., (1996), Exogenous jasmonates stimulate insect wounding in tomato plants (*Lycopersicon esculentum*) in the laboratory and field, J. Chemical Ecology, 22(10): 1767-1781.

Thayer, S. S., Choe, H. T., Rausser, S. and Huffaker, R. C., (1988), Characterization and subcellular localization of aminopeptidases in senescing barley leaves, Plant Physiol., 87: 894-897.

Thimann, K. V., (1980), The senescence of leaves, In Thimann, K. V., (Ed.), Senescence in plants, CRC Press, Florida, pp 131-156.

Thompson, J. E., (1988), The molecular basis for membrane deterioration during senescence, In Noodén, L. D. and Leopold, A. C., (Eds.), Senescence and ageing in plants, Academic Press Inc., San Diego, pp 52-77.

Traub, H. P., (1937), Methods of propagating daylilies (*Hemerocallis*) vegetatively, Herbertia, 4: 205-207.

Tucker, G. A. and Grierson, D., (1982), Synthesis of polygalacturonase during tomato fruit ripening, Planta, 155: 64-67.

Udvardy, J., Farkas, G. L. and Marre, E., (1969), On RNAse and other hydrolytic enzymes in excised *Avena* leaf tissues, Plant and Cell Physiol., 10: 375-386

Valpuesta, V., Lange, N. E., Guerrero, C. and Reid M. S., (1995), Up-regulation of cysteine protease accompanies the ethylene-insensitive scenescence of daylily (*Hemerocallis*) flowers, Plant Mol. Biol., 28: 575-582.

van Doorn, W. G., (1989), Role of physiological processes, microorganisms, and air embolism in vascular blockage of cut rose flowers, Acta Hort., 261: 27-34.

van Doorn, W. G., Harkema, H. and Song, J. S., (1995), Water relations and senescence of cut *Iris* flowers: effects of cycloheximide, Postharvest Biol. and Tech., 5: 345- 351.

van Doorn, W. G., Schurer, K. and de Witte, Y., (1989), Role of endogenous bacteria in vascular blockage of cut rose flowers, J. Plant Physiol., 134: 375-381.

van Loon, L. C., (1985), Pathogenesis-related proteins, Plant Mol. Biol., 4: 111-116.

van Staden, J., Cook, E. L. and Noodén, L. D., (1988), Cytokinins and senescence, In Noodén, L. D. and Leopold, A. C., (Eds.), Senescence and ageing in plants, Academic Press Inc., San Diego, pp 282-312.

Vierstra, R. D., (1993), Protein degradation in plants, Annu. Rev. Plant Physiol. Plant Mol. Biol., 44: 385-410.

Vierstra, R. D., (1996), Proteolysis in plants: Mechanisms and functions, Plant Mol. Biol., 32(1-2): 275-302.

Walling, L. L. and Gu Y-Q., (1996), Plant aminopeptidases: Occurrence, function and characterization, In Taylor, A. (Ed.), Aminopeptidases, R. G. Landes Comp. NY, pp 173-218.

Wang, H., Brandt, A. S. and Woodson, W. R., (1993), A flower senescence-related mRNA from carnation encodes a novel protein related to enzymes involved in phosphate biosynthesis, Plant Mol. Biol., 22: 719-724.

Warman, T. W. and Solomos, T., (1988), Ethylene production and action during foliage senescence in *Hedera helix* L., J. Exp. Bot., 39(203): 685-694.

Waters, S. P. and Dalling, M. J., (1979), Distribution and characteristics of aminoacyl β-naphthylamidase activities in wheat seedlings, Aust. J. Plant Physiol., 6: 595-606.

Waters, S. P. and Dalling, M. J., (1984), Isolation and some properties of an aminopeptidase from the primary leaf of wheat (*Triticum aestivum* L.), Plant Physiol., 75: 118-124.

Whitehead, C. S., Halevy, A. H. and Reid, M. S., (1984a), Roles of ethylene and 1-aminocyclopropane-1-carboxylic acid in pollination and wound-induced senescence of *Petunia hybrida* flowers, Physiol. Plant., 61: 643-648.

Whitehead, C. S., Halevy, A. H. and Reid, M. S., (1984b), Control of ethylene synthesis during development and senescence of carnation petals, J. Am. Soc. Hort. Sci., 109(4): 473-475.

Williams, M. H., Nell, T. A. and Barrett, J. E., (1995), Investigation of proteins in petals of potted chrysanthemum as a potential indicator of longevity, Post Harvest Biol. Tech., 5: 91-100.

Wittenbach, V. A., (1978), Breakdown of ribulose biphosphate carboxylase and change in proteolytic activity during dark-induced senescence of wheat seedlings, Plant Physiol., 62: 604-608.

Wittenbach, V. A., (1979), Ribulose biphosphate carboxylase and proteolytic activity in wheat leaves from anthesis through senescence, Plant Physiol., 64: 884-887.

Woltering, E. J., (1987), Effects of ethylene on ornamental pot plants: A classification, Sci. Hortic, 31: 283-294.

Woltering, E. J., van Doorn W., (1988), Role of ethylene in scenescence of petals-Morphological and taxonomical relationships, J. Exp. Bot., 39(208): 1605-1616.

Wolz, R. L., (1999), Strategies for inhibiting proteases of unknown mechanisms, In Sterchi, E. E. and Stöcker, W., (Eds.), Proteolytic enzymes, tools and targets, Springer-Verlag, Berlin, pp 90-108.

Woodson, W. R., (1987), Changes in protein and mRNA populations during the scenescence of carnation petals, Physiol. Plant., 71: 495-502.

Woodson, W. R., Hanchey, S. H. and Chisholm, D. N., (1985), Role of ethylene in the scenescence of isolated *Hibiscus* petals, Plant Physiol., 79: 679-683.

Woodson, W. R. and Lawton, K. A., (1988), Ethylene-induced gene expression in carnation petals, relationship to autocatalytic ethylene production and scenescence, Plant Physiol., 87: 498-503.

Wulster, G., Sacalis, J. and Janes, H., (1982), The effects of inhibitors of protein synthesis on ethylene-induced scenescence in isolated carnation petals, J. Am. Soc. Hort. Sci., 107(1): 112-115.

Zhao, Y., Botella, M. A., Subramanian, L., Niu, X., Nielsen, S. S., Bressan, R. A. and Hasegawa, P. N., (1996), The wound inducible soybean cysteine proteinase inhibitors have greater inset digestive proteinase inhibitory activities than a constitutive homolog, Plant Physiol. (Rockville), 111(4): 1299-1306.

APPENDICES

Appendix 1

Stock Solutions used in media Preparation for Tissue Culture

Table A: Murashige and Skoog (1962) Major Salts.

Chemical	Concentration (g/L)
NH_4NO_3	16.5
KNO_3	19.0
$CaCl_2.2H_2O$	4.4
$MgSO_4.7H_2O$	3.7
KH_2PO_4	1.7

Stored at 4°C.

Table B: Murashige and Skoog (1962) Minor Salts.

Chemical	Concentration (g/L)
KI	83
H_3BO_3	620
$MnSO_4.4H_2O$	2230
$ZnSO_4.7H_2O$	860
$Na_2MoO_4.2H_2O$	250
$CuSO_4.5H_2O$	2.5
$CoCl_2.6H_2O$	2.5

Stored at 4°C.

Table C: Murashige and Skoog (1962) Iron (Fe-EDTA) Stock Solution.

Chemical	Concentration (mg/L)
FeS	
$FeSO_4.7H_2O$	1390
$2Na.EDTA.2H_2O$	1865

$FeSO_4$ and EDTA were dissolved separately, mixed, the pH was adjusted to 4.8, and stored at 4°C in a dark bottle.

Table D: Murashige and Skoog (1962) Organic Stock.

Chemical	Concentration (mg/L)
Myo-insitol	10 000
Nicotinic acid	50
Pyridoxine-HCl	50
Thiamine-HCl	50
Glycine	50

Stored at 4°C in a dark bottle.

Appendix 2

Buffers

Table A: Phosphate buffer, pH 7.

Solution A: 0.1 M Potassium dihydrogen phosphate (KH_2PO_4, molecular weight 136.09, 13.61 g/L)

Solution B: 0.1 M Disodium hydrogen phosphate ($Na_2HPO_4.2H_20$, molecular weight 177.99, 17.8 g/L)

mL of solution A	mL of solution B	pH
4.0	6.0	6.98

Mixed solution A and B as described in the table and stored at 4°C.

Table B: Citrate-phosphate buffers.

Solution A: 0.1 M Citric acid (19.21 g/L)

Solution B: 0.2 M dibasic sodium phosphate (53.65 g/L of $Na_2HPO_4.7H_2O$)

mL of solution A	mL of solution B	pH
44.6	5.4	2.6
42.4	7.8	2.8
37.7	12.3	3.2
33.9	16.1	3.6
30.7	19.3	4.0
24.3	25.7	5.0
23.3	26.7	5.2
16.9	33.1	6.2
6.5	43.6	7.0

Mixed solution A and B as described in the table, diluted to a total volume of 100 mL and stored at 4°C.

Table C1: Tris HCl buffer.

Solution A: 0.2 M Tris-(hydroxymethyl)-aminomethane (Tris, molecular weight 121.14, 24.23 g/L)
Solution B: 0.1 M HCl

mL of solution A	mL of solution B	pH
25.0	10.0	8.7
25.0	2-3	9.6
25.0	Few drops	9.8

Mixed solution A and B as described in the table, diluted to a total volume of 100 mL with water and stored at 4°C.

Table C2: Tris HCl buffers for Laemmli gels.

Chemical A: 1 M Tris-(hydroxymethyl)-aminomethane (Tris, molecular weight 121.14)
Chemical B: Concentrated HCl

g of A	mL of solution B	pH
121.14	16-20	8.8
121.14	80-100	6.8

Dissolved A, adjusted the pH to required value using approximate volume of B as described in the table, diluted to a total of volume of 1000 mL with water and stored at 4°C.

Table C3: Tris HCl buffer (1 M) used for nucleic acid experiments.

Chemical A: 1 M Tris-(hydroxymethyl)-aminomethane (Tris, molecular weight 121.14)

Chemical B: Concentrated HCl

g of A	mL of solution B	pH
121.1	42.0	8.0
121.1	10.0 (approximately)	9.5

Dissolved A in 800 mL of distilled water, added solution B as described in the table, diluted to a total of volume 1000 mL with water. Diluted 1 M solution as required to get the solutions of lower strength. Stored at 4°C.

Table D: Sodium acetate buffer.

Solution A: 0.1 M Sodium acetate (molecular weight, 82.03, anhydrous 8.2 g/L)

Solution B: 0.1 M Acetic acid (molecular weight, 60.05, 5.8 mL/L)

mL of solution A	mL of solution B	pH
36	164	4.0
171	29	5.4

Mixed solutions A and B to prepare 200 mL of buffer. Stored at 4°C.

Table E: Sodium phosphate buffer.

Solution A: 0.2 M Disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$, molecular weight, 178.05, 35.61 g/L)

Solution B: 0.2 M Sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$, molecular weight 138.01, 27.6 g/L)

mL of solution A	mL of solution B	pH
6.15	43.85	6.0

Mixed solutions A and B as described in the table, diluted to a total volume of 100 mL with water and stored at 4°C.