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ETHYLENE SENSITIVITY: THE ROLES OF POLYAMINES AND SHORT-CHAIN SATURATED FATTY ACIDS IN PETUNIA AND CYCLAMEN FLOWERS

by

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THESIS

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CO-SUPERVISOR: PROF. G.H. DE SWARDT

NOVEMBER 1989
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UITREKSEL

Verouderingsverval van Petunia hybrida blomme gaan gepaard met 'n afname in die sintese van Put en Spd gedurende die vroeë stadium. Gedurende hierdie fase word Put gevorm uit L-arginien, terwyl dit in die later stadiums van verouderingsverval langs 'n alternatiewe weg gevorm word, of vrygestel word uit konjugate van Put. Bestuiwing veroorsaak dat die afname in Put en Spd versnel word. Behandeling met poli-amiene het tot gevolg gehad dat etileensensitiwiteit in onbestuifde blomme verlaag word, terwyl die uitloging van opgeloste stowwe uit die kroonweefsel verminder word. In bestuifde blomme het behandeling met poli-amiene geen invloed op die sensitiwiteit van die blomme vir etileen nie, as gevolg van die vinnige toename in sensitiwiteit wat met bestuiwing gepaard gaan. Alhoewel poli-amiene moontlik 'n rol kan speel in die beheer van etileensintese, blyk dit dat hul primêre rol in die beheer van verouderingsverval daarin geleë is dat dit in staat is om etileensensitiwiteit te verlaag. Kortketting versadigde vetsure het geen invloed op poli-amiene- en etileensintese nie, maar gee aanleiding tot 'n toename in etileensensitiwiteit as gevolg van hul destabiliserende invloed op selmembrane. Etileensensitiwiteit in Petunia blomme word beheer deur 'n wisselwerking tussen poli-amiene en kortketting.
vetsure. Poli-amiensintese neem af gelyktydig met 'n
toename in vetsuursintese. Dit gee aanleiding tot die
destabilisering van selmembrane met 'n gevolglike
toename in etileensensitiwiteit. In weefsel wat nie
sensitief is vir etileen nie, soos *Cyclamen persicum*
blomme, neem die poli-amienkonsentrasies toe gedurende
die aanvangstadium van verouderingsverval en af
gedurende die finale fases van verval. Bestuiwing het
tot gevolg dat die poli-amienkonsentrasies eers afneem
nadat die kroon afgesnoer het. Dit is onbekend wat die
presiese rol van poli-amiene in hierdie weefseis is.
ABSTRACT

Senescence of *Petunia hybrida* is accompanied by a decrease in the levels of Put and Spd during the early stages due to a decrease in the rate of Put synthesis. During this stage Put is synthesized from L-arginine, while Put is synthesized via an alternative pathway or liberated from Put conjugates during the final stages of senescence. Pollination accelerates the decrease in polyamine levels. Treatment with polyamines result in a decrease in ethylene sensitivity in unpollinated flowers and a suppression of solute leakage from isolated corolla discs. In pollinated flowers polyamines had no effect on ethylene sensitivity due to a rapid increase in the pollination-induced stimulation of sensitivity. Although polyamines could have an effect on ethylene production, their primary action in delaying senescence is related to their ability to inhibit ethylene sensitivity. Short-chain saturated fatty acids do not affect polyamine or ethylene synthesis, but result in a rapid increase in ethylene sensitivity due to their effect on destabilizing cell membranes. Polyamines and fatty acids interact to control ethylene sensitivity in *Petunia* flowers. Polyamine synthesis decreases simultaneously with the increase in fatty acid synthesis, resulting in a destabilization of cell membranes and an increase in
ethylene sensitivity. Polyamine levels increase in ethylene insensitive flowers such as Cyclamen persicum during the initial stages of senescence and decrease only during the final stages. Pollination of these flowers resulted in a decrease in polyamine levels only after abscission occurred. The exact role of polyamines in these tissues remain unclear.
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All my fellow students and friends for their motivation and encouragement

To our Devine Father without Whom nothing on earth is possible
## ANNEXURE

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC</td>
<td>1-aminocyclopropane-1-carboxylic acid</td>
</tr>
<tr>
<td>ADC</td>
<td>adenosine decarboxylase</td>
</tr>
<tr>
<td>ADP</td>
<td>adenine diphosphate</td>
</tr>
<tr>
<td>AOA</td>
<td>aminooxyacetic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenine triphosphate</td>
</tr>
<tr>
<td>AVG</td>
<td>aminoethoxyvinylglycine</td>
</tr>
<tr>
<td>D-arg</td>
<td>D-arginine</td>
</tr>
<tr>
<td>DFMA</td>
<td>difluoromethyladenine</td>
</tr>
<tr>
<td>DFMO</td>
<td>difluoromethylornithine</td>
</tr>
<tr>
<td>EFE</td>
<td>Ethylene Forming Enzyme</td>
</tr>
<tr>
<td>IAA</td>
<td>indoleacetic acid</td>
</tr>
<tr>
<td>MACC</td>
<td>malonyl-1-aminocyclopropane-1-carboxylic acid</td>
</tr>
<tr>
<td>MGBGH</td>
<td>methylglyoxal bis-(guanylhydrazone)</td>
</tr>
<tr>
<td>MTA</td>
<td>5-methylthioadenosine</td>
</tr>
<tr>
<td>MTR</td>
<td>5-methylthioribose</td>
</tr>
<tr>
<td>ODC</td>
<td>ornithine decarboxylase</td>
</tr>
<tr>
<td>Put</td>
<td>Putrescine</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>SAMDC</td>
<td>S-adenosylmethionine decarboxylase</td>
</tr>
<tr>
<td>Spm</td>
<td>Spermine</td>
</tr>
<tr>
<td>Spd</td>
<td>Spermidine</td>
</tr>
<tr>
<td>STS</td>
<td>Silver thiosulphate</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 SENESCENCE

1.1.1 What is Senescence?

Senescence is that phase in the ontogeny of a plant organ, tissue or cell which sooner or later leads to its death. Although the term ageing is often used synonymous with senescence, the two terms actually refer to two different developmental phenomena. Ageing can be defined as all those changes which occur in time without reference to death as a consequence (Medawar, 1957).

The senescence process in plants is characterized by numerous structural and metabolic changes. It appears that a wide range of growth substances are involved in the control of plant senescence. However, it is difficult to make any generalizations about the control of senescence because species differ greatly in their behaviour. The flower appears to be an excellent model system for studying the fundamental processes involved in the control of plant senescence because it has a clearly defined lifespan and because the time between
maturity and senescence is much shorter in petals than in leaves or fruit, making it possible to study such processes without recourse to artificial "senescence-inducing" treatments (Halevy and Mayak, 1977, 1979). However, it must be taken into account that the flower is a complex organ comprising of various parts, each of which may undergo different developmental rates and may interact with each other in the regulation of their senescence (Halevy and Mayak, 1977, 1979).

In general flowers can be divided into two groups with reference to their senescence as to i) their relationship to ethylene and ii) the cause of their life termination (Whitehead and Halevy, 1989). Flowers can further be divided into different groups with reference to their method of senescence. In one group senescence is characterized by corolla abscission whilst still fully turgid, e.g. Digitalis purpurea (Stead and Moore, 1983). In yet another group of which Dianthus caryophyllis is a good example, petal senescence is accompanied by a loss in turgidity, inrolling and browning. In these flowers senescence does not lead to petal abscission. Petunia hybrida is representative of a group of flowers which show some characteristics of both the above mentioned groups. In these flowers the corolla wilts and may later abscind or loosen its attachment to the recepticle.
(Gilissen, 1977; Nichols and Frost, 1985). Another feature of senescence in *Petunia* is that colour changes may occur in the corolla during senescence, making it possible to visually follow the progress of senescence by simply noting the rate of corolla blueing (Whitehead *et al.*, 1984a; Whitehead and Halevy, 1989). Aside from these characteristics, other advantages of the *Petunia* flower as a model system for studying senescence is that the flower has a single, uniform, unattached corolla and a small, well defined stigma with a single, relatively long style to facilitate the study of pollination-induced senescence.

Senescence of *Petunia* flowers is associated with a climacteric rise in ethylene production during the final stages regardless of whether it is caused naturally or by pollination or wounding of the stylar tissue (Whitehead *et al.*, 1984a). Pollination-induced senescence of the corolla is a good example of a case where a process which occurs in one part of an organ, affects the development of another part of the same organ. Pollination precedes corolla senescence and accelerates the senescence process in the corolla (Lovell *et al.*, 1987a,b; Nichols and Frost, 1985; Whitehead *et al.*, 1984a). The acceleration of corolla senescence caused by pollination or mechanical wounding of the stigma, is associated with an acceleration in
both the rate of wilting and the onset of the climacteric rise in ethylene production (Whitehead et al., 1984a), indicating that a signal or stimulus is produced in the style which is then transported to the corolla where it stimulates ethylene production and senescence. It is believed that 1-aminocyclopropane-1-carboxylic acid (ACC) the immediate precursor to ethylene (Adams and Yang, 1979), may be the signal which stimulates ethylene production in the corollas of pollinated flowers (Reid et al., 1984). Following pollination and penetration of the stigma by the growing pollen-tubes, ACC is produced in the stylar tissue in response to the "wounding" of the style by the growing pollen-tubes. This ACC is then transported to the corolla where it is rapidly converted to ethylene which may result in the autocatalytic stimulation of ethylene production and an acceleration in the rate of corolla senescence (Hoekstra and Weges, 1986; Lovell et al., 1987a,b; Nichols and Frost, 1985; Whitehead et al., 1984a).

1.1.2 Senescence Signals

As mentioned above, ethylene may play an important role in accelerating senescence in pollinated flowers. The involvement of ethylene in pollination-induced senescence of Petunia flowers, is clearly demonstrated
by the observation that treatment with an inhibitor of ethylene action such as silver thiosulphate (STS) (Veen, 1979) prior to pollination, effectively delays their senescence (Lovell et al., 1987b; Whitehead et al., 1984a). The acceleration of senescence and ethylene production following pollination indicates that some stimulus or signal is produced in the stylar tissue and transported to the corolla where it then stimulates ethylene production (Lovell et al., 1987a; Whitehead et al., 1984a).

The plant growth regulator indoleacetic acid (IAA) has been implicated in the stimulation of ethylene synthesis in plant tissue (Morgan and Hall, 1964). According to Burg and Dijkman (1967) IAA may be the signal responsible for stimulating ethylene production in pollinated flowers. However, this possibility is ruled out by the observation made by Strauss and Arditti (1982) and Reid et al. (1984) that auxin movement is very slow in the stylar tissues of orchid and carnation flowers. Furthermore, application of IAA to the stigma or injection of IAA into the ovaries of unpollinated Petunia flowers had no effect on ethylene production and did not stimulate senescence of the corolla (Lovell et al., 1987b; Whitehead and Halevy, 1989).
Pollination results in a dramatic increase in ethylene production in the gynoecium of certain flowers (Whitehead et al., 1983a,b; 1984a). However it appears that this ethylene is not responsible for the wilting of the corolla, since removal of gynoecium-induced ethylene does not prevent accelerated corolla senescence (Hoekstra and Weges, 1986; Reid et al., 1984). Another signal must therefore be produced in the gynoecium which will result in the stimulation of ethylene production and senescence in the corolla. Results from various research workers indicate that ACC may be the transmitted stimulus produced in the styles of pollinated flowers. Raised ACC levels were found in different floral parts of pollinated carnation flowers (Nichols et al., 1983; Hsieh and Sacalis, 1986). It appears that ACC is transported from the styles to the corolla where it is converted to ethylene (Reid et al., 1984; Whitehead et al., 1984a; Hoekstra and Weges, 1986). Although Cyclamen flowers produce very little ethylene during natural senescence, pollination also results in a dramatic increase in ethylene production by the corolla (Halevy et al., 1984). It seems reasonable to suggest that a similar mechanism may be operating in these flowers.
1.1.3 The "Sensitivity Factor"

The idea that the sensitivity of plant tissues to a plant growth regulator is a critical factor in the development of the plant and that this sensitivity may change under certain conditions is not a new one, but has been suggested more than fifty years ago by Went and Thimann (1937). However, more recent publications by Trewavas (1981, 1982) has renewed interest in this phenomenon. An increase in ethylene sensitivity with age is known to occur in many plant tissues (Brady, 1987; Halevy and Mayak, 1981; Whitehead and Halevy, 1989).

In Petunia and Cyclamen it appears that over and above the stimulation of ethylene production, pollination also results in a marked increase in the sensitivity of the corolla to ethylene (Halevy et al., 1984; Whitehead et al., 1984a). Halevy et al. (1984) and Halevy and Whitehead (1989) suggested that aside from the production of ACC, a "sensitivity factor" is also produced in the stylar tissue and transported to the corolla of pollinated flowers where it results in the stimulation of ethylene sensitivity. The production of such a "sensitivity factor" is independant of ACC, since injection of ACC or AVG into the ovaries of unpollinated and pollinated flowers has no effect on
the development of ethylene sensitivity in the corolla (Whitehead and Halevy, 1989). In their studies on the nature of the "sensitivity factor", Whitehead and Halevy (1989) discovered that short-chain saturated fatty acids ranging in length from C₆ to C₁₀ may be the stimulus responsible for the increase in ethylene sensitivity in the corolla. Their results indicate that these acids are produced via the acetate pathway in the styles of pollinated flowers. Following their production in the styles, these acids are then transported to the corolla where it induces an increase in ethylene sensitivity, possibly by their action on cell membranes. It has indeed been shown that treatment of ripening bananas with these acids may result in an increase in ethylene binding (Bossé and Whitehead, unpublished results). It appears that nonanoic acid (C₉) is the most abundant acid produced by the styles of pollinated flowers (Vasiljevic and Whitehead, unpublished results). Indications are that these acids may also be involved in stimulating ethylene sensitivity in unpollinated flowers during senescence (Whitehead and Halevy, 1989). It is clear from the above discussion that the production of both an ethylene "stimulating factor" (ACC) and a "sensitivity factor" (short-chain saturated fatty acids) is required
for the reaction of the corolla to pollination. Production of anyone of these factors without the other will not have the same effect on corolla senescence.

1.2 POLYAMINES

The term polyamines collectively refers to a group of substances which includes the diamine putrescine (Put) and the polyamines spermidine (Spd) and spermine (Spm). Production of polyamines is a requirement for normal growth and development and is not a consequence of growth. Because of their universal occurrence, it is suggested that polyamines fulfill an important role in relation to plant hormone action, senescence, light reactions and stress (Smith, 1985). Amongst the many roles ascribed to polyamines are the following:

i. a new class of growth regulators (Smith, 1985)

ii. second messengers to plant hormones (Smith, 1985)

iii. a source of carbon and nitrogen in the plant (Galston, 1983; Kumar and Thorpe, 1989; Slocum et al., 1984).

iv. protectors against ozone (O₃) damage (Bors et al., 1989)

Polyamines are also implicated as anti-senescence agents, since they are able to inhibit fruit ripening (Winer and Apelbaum, 1986), senescence (Slocum et al.,...
1984) and ethylene production (Apelbaum et al., 1981b; Ben-Arie et al., 1982; Even-Chen et al., 1982; Suttle, 1981).

1.2.1 Polyamine Biosynthesis

Polyamines are synthesized from L-Arginine and S-adenosylmethionine (SAM) as shown in Fig 1. SAM in turn, is synthesized from the amino acid methionine which is formed via the recirculation of 5-methyl-thioadenosine (MTA) (Kushad et al., 1988). In climacteric tissues the conversion of MTA to methionine occurs during the pre-climacteric and climacteric stages (Adams and Yang, 1977). During this process MTA is actively metabolized to 5-methylthioribose (MTR) and MTR-1-P by the removal of adenosine from MTA and the subsequent phosphorylation of MTR by the conversion of ATP to ADP. These reactions are catalized by the enzyme MTA nucleosidase and MTR kinase respectively. Increases in the activities of both these enzymes are presented as evidence that MTR and MTR-1-P are intermediates involved in the regeneration of methionine and therefore indirectly in the synthesis of SAM (Kushad et al., 1988) (Fig. 1).
Several enzymes could be involved in the biosynthesis of polyamines in plants from SAM and L-Arginine. These include ornithine decarboxylase (ODC), arginine decarboxylase (ADC), S-adenosylmethionine decarboxylase (SAMDC) and Spd synthase (Christ et al., 1989). The enzymes ADC and ODC are key enzymes in the polyamine biosynthetic pathway. However, it appears that ADC is more important than ODC in this process since its activity is very high in tissues responding to growth stimuli (Altman et al., 1982). Recent evidence suggests that polyamine biosynthesis may be controlled by either ADC or ODC activity. It is possible that these enzymes may be active at different stages in the development of the plant (Slocum et al., 1984).

According to Smith (1985) Put is mainly synthesized from arginine via the polyamine, agmatine. However, Altman (1982b) and Kaur-Sahweny et al. (1982a) suggested that the formation of Put in mung bean and oat leaves occurs directly via the decarboxylation of ornithine. Ikerson et al., (1985) proposed that Put synthesis may occur either via the decarboxylation of arginine or ornithine by the enzymes ADC and ODC respectively. According to Kushad et al. (1988) the major pathway for polyamine synthesis during early fruit development, occurs through the decarboxylation of ornithine. However, it appears that the activity of
this pathway during early fruit development is relatively insignificant. Although ornithine is implicated as a direct precursor for Put, it must be taken into account that ornithine is also incorporated into arginine and citrulline which may also indirectly serve as precursors of ethylene (Smith, 1984).

It is evident from the above discussion that several pathways may exist in plant tissues for the biosynthesis of Put, the first product in the synthesis of the polyamines Spd and Spm via the addition of propylamine groups from SAM (Bowman et al., 1973). Arginine, ornithine and citrulline have all been mentioned as possible precursors for Put. However, it appears that arginine may be the major component from which Put is synthesized in plants (Sevyakova, 1981). Polyamine levels are not decreased by the combined use of inhibitors of ADC and ODC, indicating that the synthesis of Put may occur through a completely different pathway, or that plants may compensate for the reduced availability of these enzymes by increasing their synthesis (Felix and Harr, 1989).
Bowman et al. (1973) have suggested the following sequence for the production of polyamines in plants:

\[
\text{Put} \rightarrow \text{Spd} \rightarrow \text{Spm} \rightarrow \text{SAM} \rightarrow \text{MTA}
\]

It appears that both SAMDC and propylamine transferase are involved in catalyzing the biosynthesis of Spd from Put and SAM. SAM serves as a source for propylamine groups for the synthesis of Spd and Spm from Put (Roberts et al., 1984; Miyazaki and Yang, 1987a).
Inhibition of ethylene synthesis may result in an increase in the levels of the Spd, possibly because of an increase in the availability of propylamine groups derived from SAM (Roberts et al., 1984). The biosynthesis of propylamines is thought to be closely related to the regulation of ACC synthesis. Results obtained by Katoh et al. (1987) indicate that a decrease in the levels of ACC results in an increase in polyamine levels and vice versa.

1.2.3 Inhibitors of Polyamine Synthesis

Polyamine concentrations reach a peak during the early stages of fruit development and decreases to low levels during the later stages of development (Kushad et al., 1988). The natural onset of senescence is a result of a decrease in the endogenous polyamine levels. Treatment with exogenous polyamines may result in a delay in senescence of excised leaves. It appears that this response may occur in all plant tissues (Slocum et al., 1984). Senescence of carnation flowers is accompanied by a sharp rise in ethylene production during the final phases. This increase in ethylene production is associated with an increase in the levels of endogenous Put. However, the levels of Spd and Spm remain
unchanged during this period. It appears that the activity of either Spd synthase or SAMDC may be the limiting factor in this process (Roberts et al., 1984).

The use of specific inhibitors of polyamine synthesis could be a useful tool in studying the mechanisms involved in modulating polyamine levels during plant senescence. Examples of inhibitors of polyamine synthesis are D-arginine (D-arg), methylglyoxal bis-(guanylhydrazone) (MGBGH), difluoromethylornithine (DFMO) and difluoromethylarginine (DFMA). D-arginine is an inhibitor of Put synthesis. DFMO specifically inhibits ODC activity while DFMA inhibits ADC activity. MGBGH acts as an inhibitor of SAMDC activity in plant tissues (Hiatt et al., 1986; Roberts et al., 1984).

Inhibitors of pyridoxal phosphate mediated enzymes such as aminooxyacetic acid (AOA) and aminoethoxyvinylglycine (AVG) may also affect polyamine synthesis by inhibiting pyridoxal phosphate enzymes such as SAMDC and ADC (Christ et al., 1989; Dai et al., 1982; Roberts et al., 1984). These inhibitors are however not specific for polyamine enzymes and may also inhibit other enzymes which require pyridoxal phosphate as a co-factor such as ACC synthase, an enzyme involved in the synthesis of ethylene (Yu et al., 1979a).
1.2.4 Growth Regulation by Polyamines

Polyamines are involved in the stimulation and modulation of growth processes in plants (Smith, 1985). It appears that hormonal responses are accompanied by changes in the metabolism of polyamines and that polyamines may affect plant hormone action at the membrane level by competing for membrane associated hormone binding sites or by counteracting hormone-induced changes in membrane permeability (Slocum et al., 1984). Furthermore, cell divisions are associated with high activities of enzymes involved in polyamine synthesis such as ODC and SAMDC (Kaur-Sawheny et al., 1982b). Cells in division are rich in Spd and Spm (Galston, 1983) and the levels of these compounds continually increase during the division process. This promotion of growth by polyamines appear to be associated with their ability to stabilize nucleic acids and cell membranes (Slocum et al., 1984).

The application of stimulatory growth hormones such as auxins (Bagni et al., 1981), gibberellins (Dai et al., 1982) and cytokinins (Suresh et al., 1978) to plant tissues results in an increase in polyamine biosynthesis and titer (Galston, 1983). The synthetic auxin 2,4-D as well as cytokinins, act by activating
the synthesis of endogenous polyamines (Slocum et al., 1984; Suresh et al., 1978). The promotion of growth in dwarf peas by treatment with gibberellins is also accompanied by an increase in polyamine titers and ADC activity (Dai et al., 1982).

1.3 ETHYLENE

The discovery that ethylene acts as a plant growth regulator dates back to the previous century when it was observed that leaking illuminating gas was responsible for accelerating leaf senescence and abscission. It was later discovered that ethylene is the active principle in natural gas and ethylene was positively identified as a natural plant product by Gane in 1934 (Abeles, 1973). Ethylene is a gaseous plant hormone and is active in very small quantities. All plant tissues produce ethylene during their growth and development (Baile, 1954). Young climacteric fruit produce very little ethylene, but ethylene production increases dramatically during the ripening process. This observation lead to the suggestion by Burg and Burg (1965) that ethylene is a natural endogenous ripening hormone. Ethylene is regarded as a plant
hormone because it is a natural plant product and because it is able to regulate plant growth and development (Sisler and Yang, 1984).

Ethylene production in climacteric tissues is higher than in non-climacteric tissues. Climacteric tissues are characterized by a low rate in ethylene production during the pre-climacteric stage. However, in these tissues ethylene production rises sharply during the climacteric stage (Sisler and Yang, 1984). The longevity of climacteric tissues is decreased when exposed to ethylene. In carnation flowers longevity is correlated with the amount of ethylene produced by the tissue and the dose of ethylene received when treated with exogenously applied ethylene (Barden and Hanon, 1972).

Two groups of flowers can be distinguished with relation to their ethylene production and sensitivity: a) Those that show a climacteric rise in ethylene production during senescence and is sensitive to ethylene and b) those in which ethylene does not play a substantial role in accelerating their senescence (Halevy and Whitehead, 1989).
1.3.1 Ethylene Biosynthesis

In higher plants ethylene is produced from the amino acid, methionine via a series of enzyme catalyzed biochemical reactions. The first step in the synthesis of ethylene, is the production of SAM from methionine. This is followed by the conversion of SAM to ACC by the enzyme ACC synthase. The final step in this pathway is the oxidation of ACC to ethylene by the ethylene forming enzyme (EFE) (Adams and Yang, 1979). It appears that the EFE is a membrane bound enzyme and that the conversion of ACC to ethylene is a membrane associated process (Apelbaum et al., 1981a). ACC appears to be the key precursor in the pathway. The presence of oxygen is absolutely essential for the production of ethylene from ACC. Under aerobic conditions ACC is rapidly converted to ethylene, indicating that this conversion is an oxygen-dependant step in the synthesis of ethylene (Sisler and Yang, 1984). Ethylene synthesis is stimulated by indoleacetic acid (IAA) due to an increase in the activity of ACC synthase. Although ethylene synthesis is not affected directly by the presence of CO₂, ethylene action is retarded due to the competition between the two gasses for the ethylene binding sites (Sisler and Yang, 1984).
Ethylene originates from the C₃ and C₄ positions of methionine. The methylthio-group (CH₃S) is retained and recycled via SAM, MTA and MTR back to methionine to sustain a steady supply of methionine when the endogenous levels of this amino acid is low (Adams and Yang, 1979). Although methionine is normally present in low quantities in plant tissues, its availability does not appear to be limiting in the production of ethylene. Several mechanisms have been suggested to be involved in the control of ethylene synthesis. These include changes in the activities of ACC synthase (Adams and Yang, 1979) and the EFE (Yang, 1980), as well as the conjugation of ACC into malonyl-ACC (MACC) (Hoffman et al., 1983). It appears that ethylene production is controlled by the rate of ACC production and not by the rate of the conversion of ACC to ethylene (Cameron et al., 1979). According to Whitehead et al., 1984a the EFE is present at all stages during the senescence of carnation petals, although the activity of this enzyme increases simultaneously with the increase in ACC content of the petals. Any ACC present in the petal tissue would therefore immediately be converted into ethylene. From these and other studies it appears that the conversion of SAM to ACC is the rate-limiting step in ethylene biosynthesis during plant senescence (Nichols et al., 1983; Whitehead et al., 1984a; Yu et al., 1979a,b). This step is
controlled by the activity of ACC synthase. Treatment with inhibitors of ACC synthase, such as AVG and ADA will result in a suppression of ethylene biosynthesis and an increase in the longevity of the tissue (Yu et al., 1979a).

In senescing carnation petals ethylene production is controlled by the availability of ACC. ACC levels remain low during the pre-climacteric phase, but increases markedly simultaneously with the climacteric rise in ethylene production (Nichols et al., 1983; Whitehead et al., 1984a). However, in pollinated carnation and Petunia flowers autocatalytic ethylene production in the corolla is stimulated by the transportation of ACC from the gynoecium. This ACC is produced in response to the "wounding" of the stylar tissue by the growing pollen-tubes (Hoekstra and Weges, 1986; Nichols et al., 1983; Nichols and Frost, 1985; Reid et al., 1984; Whitehead et al., 1983a and 1984a). Although the pollen of several species contain substantial amounts of ACC, pollen ACC appears not to be involved in the stimulation of autocatalytic ethylene production in the corollas of pollinated flowers (Hoekstra and Weges, 1986; Whitehead et al., 1983a).
1.3.2. **Ethylene Binding**

Like all other plant hormones, ethylene has to bind to a specific receptor molecule before plant tissues will respond to its presence. This receptor is highly specific and has a high affinity for ethylene. It provides plant cells with the capacity to distinguish ethylene from any other molecules to which they are exposed. The receptor molecule appears to be membrane-bound copper-containing protein (Thompson et al., 1983) located in the endoplasmic reticulum and plasmalemma of the cell (Evans et al., 1982). Although ethylene binding sites have been discovered in several plant species, the proteins involved have been characterized only in *Phaseolus vulgaris* and *P. aureus* (Hall, 1986). However, the exact nature of these proteins is still not clarified. Although Beyer (1979) proposed that ethylene metabolism is an integral part of the ethylene action mechanism, it appears that the enzymes involved in ethylene metabolism are unlikely to function as ethylene receptors (Sanders et al., 1989b). According to Sanders et al. (1989b) and Sisler and Yang (1984), ethylene metabolism is not directly linked to the mode of action of this growth regulator.
Direct evidence for the mechanism of ethylene binding is not easy to find due to the difficulties encountered in measuring ethylene binding (Sisler, 1979). To measure ethylene binding in plant tissues radio-labelled ethylene is replaced with unlabelled ethylene. However, ethylene metabolism may interfere with the binding assay, giving rise to incorrect estimations of the amount of binding sites present in the tissue. This problem was, however, solved in a recent study by Sanders et al. (1989a) in which they describe several techniques devised to distinguish between bound \textsuperscript{14}C-ethylene and \textsuperscript{14}C-ethylene metabolites. Although the exact nature of the mechanisms involved in ethylene binding remains unclear, it is evident that the process is not non-physiological in nature, but that binding of ethylene is to a specific physiological receptor (Sisler and Wood, 1987).

1.3.3. Compartmentation of Ethylene and Ethylene Synthesis

Accumulating evidence suggest that different steps in the synthesis of ethylene may occur at different sites within the cell. Since the discovery by Adams and Yang (1979) that ACC is the immediate precursor for ethylene which is synthesized in the cytoplasm, various studies have been conducted in an effort to assign the ethylene
formation system to a particular subcellular entity. Although it is generally accepted that ACC synthesis is localized in the cytoplasm (Adams and Yang, 1979; Guy and Kende, 1984a) some confusion exists regarding the subcellular site for the conversion of ACC to ethylene. Evidence from different studies suggest that a number of different subcellular organelles may be involved in the conversion of ACC to ethylene. Studies by Mayak et al. (1981) indicate that the EFE is present in microsomal membranes obtained from carnation flowers and that these membranes may be the locality for the production of ethylene from its immediate precursor, ACC. According to Vinkler and Apelbaum (1985) the mitochondria is the obvious site for this conversion process to occur. Their results suggest that the conversion of ACC to ethylene is associated with the inner mitochondrial membrane and that the transport of ACC across the intact mitochondrial membrane may be rate-limiting in this process. However, recent studies by Bouzayen et al. (1986), Guy and Kende (1984a,b) and Tophof et al. (1989) indicate that ACC is predominantly present in the vacuole and that ACC conversion to ethylene occurs in the tonoplast. Furthermore, MACC has also been shown to accumulate in the vacuole (Bouzayen et al., 1986; Tophof et al., 1989). However, no specific carrier for ACC exists in the tonoplast while MACC transport across the tonoplast is catalized by
a transport system which is also responsible for the transport of malate into the vacuole (Tophof et al., 1989). It appears that the vacuole could be one, but perhaps not the only organelle involved in ethylene formation from ACC. Aside from the compartmentation of ethylene synthesis, ethylene itself also appears to be compartmented in the cell (Jerie et al., 1979). However, the nature of ethylene compartmentation remains uncertain.

1.4. POLYAMINES AND PLANT SENESCEENCE

Evidence now exists which indicates that natural polyamines may stabilize cell membranes and retard plant senescence. Exogenous application of polyamines may delay senescence in excised leaves or leaf segments of a number of monocots and dicots. Treatment with polyamines results in an inhibition of RNase and protease activity and a delay in chlorophyll degradation observed during senescence. Furthermore, senescence of dark incubated leaf discs is characterized by a decrease in ADC activity and polyamine titers. This decrease can be reversed by transferring the leaf discs to light (Kaur-Sawhney et al., 1982a). These effects have been attributed to the
binding of polyamines to membranes where it may exert an effect on ethylene biosynthesis (Slocum et al., 1984).

1.4.1. Polyamine and Ethylene Interactions

As indicated in the previous sections, the polyamines Spd and Spm, and ethylene share SAM as a common precursor in their biosynthetic pathways. It therefore seems logical to assume that changes in the synthesis of polyamines may exert an effect on ethylene biosynthesis or vice versa. Both ethylene and polyamines are known to have regulatory roles in plant development which may affect senescence. Ethylene may accelerate senescence in certain species while polyamines have a protective role which may result in a delay in the senescence processes (Downs and Lovell, 1986). Competition for SAM between the polyamine and ethylene pathways may thus control senescence by a mechanism of feedback regulation (Even-Chen et al., 1982; Roberts et al., 1984; Katoh et al., 1987).

According to Apelbaum et al. (1981a) and Suttle (1981), treatment with ethylene results in an inhibition of polyamine synthesis in plants. Ethylene synthesis may, however, also be inhibited by polyamines due to a decrease in the synthesis of ACC (Even-Chen et al.,
1982; Fuhrer et al., 1982), and an inhibition of the conversion of ACC to ethylene (Apelbaum et al., 1981b; Fuhrer et al., 1982; Suttle, 1981). The decrease in ACC synthesis may be the result of an increase in the synthesis of Spd and Spm, which could decrease the amount of SAM available for ACC synthesis (Roberts et al., 1984).

Although some workers have suggested that the inhibition of ethylene synthesis by polyamines may be caused by a decrease in ACC synthesis, evidence exists which indicate that polyamines have very little effect on ACC synthesis and that they exert their effect primarily by controlling the conversion of ACC to ethylene. According to Miyazaki and Yang (1987b), polyamines are weak inhibitors of ACC synthase and their role in regulating ACC synthase activity in vivo is very limited. Furthermore, Saftner (1989) observed an increase in ACC content in tomato pericarp tissue after treatment with polyamines. He suggested that polyamines have a greater inhibitory effect on ACC catabolism than on ACC formation. An accumulation of ACC was also observed in oat leaves after treatment with polyamines despite of a decrease in ACC synthesis, indicating that polyamines may act by suppressing the conversion of ACC to ethylene (Fuhrer et al., 1982). According to Ben-Arie et al. (1982) polyamines may
inhibit the conversion of ACC to ethylene through ionic interactions with membrane lipids, thus modulating the activity of the membrane-bound ethylene forming complex.

It appears that ACC is synthesized in the cytoplasm and transported to the vacuole where it is converted to ethylene (Guy and Kende, 1984a,b; Tophof et al., 1989). It may therefore be possible that ethylene production by polyamines could be regulated not only by directly affecting the conversion of ACC to ethylene, but also by affecting ACC transport and retention within the vacuole. Experiments conducted by Saftner (1989) indicate that polyamines may indeed inhibit ethylene production by inhibiting the transport of ACC into the vacuole. It is known that the inhibition of ethylene production by polyamines could be reversed by high concentrations of Ca²⁺ (Apelbaum et al., 1981b; Fuhrer et al., 1982) and that Ca²⁺, by itself, could stimulate ethylene production in plant tissues (Mattoo and Anderson, 1984). This effect of Ca²⁺ on ethylene synthesis could be explained by the observation that Ca²⁺ strongly stimulates ACC uptake into the vacuole and ethylene synthesis. Ca²⁺ also counteract the inhibitory effects of polyamines on both ACC uptake into the vacuole and ethylene synthesis (Saftner, 1989).
In summary it can be said that polyamines inhibit ethylene biosynthesis by inhibiting ACC formation and ACC conversion to ethylene. The latter appears to be the major process involved in the regulation of ethylene production by polyamines. It appears that polyamines exert their effect on ACC conversion to ethylene by their interaction with cell membranes which could, in turn, affect the ethylene forming system as well as the movement of ACC to the site of its conversion to ethylene.

1.4.2. Polyamines and Membrane Permeability

As indicated previously, inhibition of senescence by polyamines may be related to their rapid binding to cellular membranes where they exert anti-senescent properties by inhibiting the conversion of ACC to ethylene (Slocum et al., 1984). It therefore seems appropriate to look at the effect of polyamines on membrane properties and the interaction between polyamines and membrane lipids.

Senescence of plant tissues is accompanied by a marked increase in membrane permeability as shown by Suttle and Kende (1980) with isolated Tradescantia petals. The ethylene stimulated increase in membrane permeability
in these flowers, is characterized by a great loss of membrane phospholipids. It appears that ethylene does not affect membrane permeability directly, but that the gas exerts its effect on permeability through its effect on cellular metabolism. According to Naik and Srivastava (1978) polyamines may inhibit the senescence-induced destabilization of cell membranes which gives rise to an increase in membrane permeability. The stabilizing role of polyamines is demonstrated by their ability to regulate ionic permeability in membranes (Shevyakova, 1981). Due to their polycationic nature polyamines may bind to the negatively charged headgroups of phospholipids present in cytoplasmic membranes and the plasmalemma. By doing so, polyamines may have a stabilizing effect on these membranes (Naik and Srivastava, 1978; Roberts et al., 1986), thus preventing ion leakage from the cell (Srivastava and Smith, 1982). This stabilizing effect of polyamines on cell membranes may slow down the senescence processes in the cell (Smith, 1985). The attachment of polyamines to cell membranes may also retard the conversion of ACC to ethylene (Fuhrer et al., 1982). It is unlikely that the stabilizing effect of polyamines is mediated via its interaction with nucleic acid, since their action on membrane permeability is immediate. It appears that membrane stabilization is brought about by a direct ionic
interaction between the polyamines and membrane components (Srivastava and Smith, 1982; Slocum et al., 1984).

Senescence of carnation petals is accompanied by an increase in membrane microviscosity, possibly due to an increase in sterol:phospholipid ratio caused by the selective loss of membrane phospholipid (Thompson et al., 1982). This increase in microviscosity is accelerated by treatment with ethylene and delayed by inhibition of ethylene synthesis and treatment with STS. According to Ben-Arie et al. (1982) polyamines may act by stabilizing membrane fluidity, preventing the natural increase in membrane microviscosity. It appears that the polyamines Spd and Spm are more effective than the diamine Put in reducing membrane fluidity (Roberts et al., 1986). Roberts et al. (1986) suggested that treatment with polyamines could result in a nonspecific membrane rigidification incurred when polyamines act as polyvalent cations and associate with the negatively charged headgroups of the bilayer phospholipid. However, in their studies on the effect of polyamines on K+ and H+ efflux through the plasmalemma, De Agazio et al. (1988, 1989) concluded that the effect of polyamines on the plasmalemma is to be ascribed to a specific interaction between polyamines with the plasmalemma and not to a more nonspecific membrane
rigidification. It remains to be clarified whether polyamines act directly by interacting with the plasmalemma or through the products of their oxidative catabolism. It is also possible that both may act simultaneously on the membrane (De Agazio et al., 1989).

Since the activity of the EFE is sensitive to membrane disturbances (Apelbaum et al., 1981a; Mattoo and Aharoni, 1988), it is possible that polyamines and Ca$^{2+}$ may exert their inhibiting effect on ethylene synthesis through their ability to stabilize cellular membranes (Roberts et al., 1986; Slocum et al., 1984) while at the same time inhibiting the transport of ACC across the tonoplast to the site of its conversion to ethylene (Saftner, 1989).

1.5 Short-Chain Saturated Fatty Acids

Sensitivity to ethylene varies greatly in the flowers of different plant species. Petunia and Dianthus flowers are sensitive to ethylene and treatment with ethylene or pollination will increase their sensitivity and accelerate their senescence (Maxie et al., 1973; Nichhols, 1966; Whitehead et al., 1984a,b; Whitehead and Halevy, 1989). However, some plants are more sensitive to ethylene than others (Sisler and Yang,
1984). *Cyclamen* flowers, for example, produce very little ethylene and even aged flowers do not respond to exogenous ethylene (Halevy et al., 1984). Pollination however, results in an increase in ethylene synthesis and a stimulation of ethylene sensitivity. It is clear that two signals are produced in the gynoecium of pollinated flowers which are transported to the corolla where one induces ethylene synthesis and the other an increase in ethylene sensitivity. It appears that the production of the two signals is not directly related to each other, since inhibition of ethylene synthesis by treatment with AVG does not inhibit the increase in ethylene sensitivity (Halevy and Whitehead, 1989).

In a recent study on the nature of the sensitivity signal, Whitehead and Halevy (1989) discovered that short-chain saturated fatty acids ranging in chain length from C₆ to C₁₀ are produced in the stylar tissue of pollinated *Petunia* flowers via the acetate pathway. These acids are rapidly transported to the corolla where they accumulate during the early stages of pollination-induced senescence. Treatment of unpollinated flowers with these acids by application to their stigmas, results in an increase in ethylene sensitivity similar to that observed in pollinated flowers. These fatty acids were also detected in the corollas of unpollinated flowers. In these flowers an
increase in the levels of fatty acids during the early stages of senescence is accompanied by a simultaneous increase in ethylene sensitivity. It appears that short-chain saturated fatty acids are responsible for the increase in ethylene sensitivity in senescing Petunia flowers and that these acids may act as the "sensitivity factor". Similar results were obtained with carnation flowers where nonanoic acid (C9) appears to be the most abundant fatty acid produced by the tissue (Vasiljevic and Whitehead, unpublished data).

Although the exact mode of action of these fatty acids in increasing ethylene sensitivity is still unknown, it seems possible that the acids may act by changing the physical properties of cellular membranes. Short-chain saturated fatty acids may affect membrane permeability in several plant tissues (Jackson and Taylor, 1970). According to Jackson and Taylor (1970) and Stewart and Berrie (1979) the plasmamembrane is the site of action for these acids. Incorporation of the fatty acids into membranes may cause changes in membrane fluidity resulting in a modification in the activities of exchange systems and an increase in membrane permeability. It appears that the reaction of plant tissues to short-chain saturated fatty acids, is dependent on the number of carbon atoms present in the molecule. In germinating seeds, C8 and C9 acids appear
to be the most effective in increasing ion leakage from the tissue and inhibiting germination of the seeds. In some cases this inhibition can be counteracted by treatment with cytokinins and gibberellins (Babiano et al., 1984; Berrie, 1979; Berrie et al., 1975; Stewart and Berrie, 1979).

From the above discussion it appears that both polyamines and short-chain saturated fatty acids may act by modulating membrane properties which in turn may result in changes in the sensitivity of plant tissues to ethylene. Polyamines could possibly play an important role in stabilizing cellular membranes which results in a suppression of ethylene synthesis and sensitivity, while short-chain saturated fatty acids may act by destabilizing membranes which could result in an increase in ethylene sensitivity. This increase in sensitivity is, however, not dependant on an increase in ethylene synthesis. The purpose of this study was, therefore, to investigate changes in polyamine levels and composition in Petunia hybrida and Cyclamen persicum flowers, as well as to examine different interactions between polyamines and fatty acids in an attempt to clarify their possible roles in controlling ethylene sensitivity in pollinated and unpollinated flowers.
Figure 1a: Metionine pathway of ethylene biosynthesis (Adams and Yang, 1977).
Figure 1b: Recirculation of MTA via the methionine pathway. Derived from Miyazaki and Yang (1987a).
METHIONINE → 2-oxo-4 methylthiobutanoic acid

SAM → MTR-1-P

MTR KINASE

MTA NUCLEOSIDASE

ACC SYNTHASE

ACC → MTA

C2H4
Figure 1c: Putrescine biosynthetic pathway via L-arginine and L-ornithine (Altman et al., 1982).
GLUTAMIC ACID

L-ornithine

Citrulline

L-arginine

L-argatine

N-carbamyl putrescine

Putrescine

Spermidine; Spermine

ODC (ORNITHINEDECARBOXYLASE)

ADC (ARGININEDECARBOXYLASE)
Figure 1d: Polyamine- and ethylene biosynthetic pathways, showing points of inhibition (Roberts et al., 1984)
CHAPTER 2

POLYAMINES IN PETUNIA HYBRIDA
AND CYCLAMEN PERSICUM

2.1 INTRODUCTION

Polyamines play an important role in regulating plant growth and development. It appears that polyamine concentrations are at a peak during the early stages of development and cell division (Galston, 1983; Kushad et al., 1988) and decrease as maturation of the tissue proceeds (Kushad et al., 1988). According to Slocum et al. (1984) the natural onset of senescence is the result of this decrease in polyamine levels. It is clear that polyamines are associated with the stimulation of plant growth and development (Slocum et al., 1984; Smith, 1985). This stimulation is presumably caused by their ability to bind to and stabilize cellular membranes (Slocum et al., 1984).

The synthesis of polyamines may occur via two different biosynthetic pathways. Polyamines may be synthesized from arginine via the enzyme ADC or from ornithine via the enzyme ODC (Felix and Harr, 1989; Ickerson et al., 1985). According to Kushad et al. (1988) polyamines are synthesized from ornithine during the early stages.
of development. However, the contribution of this pathway appears to be negligible during the later stages of development. Some contradiction exists in the literature regarding the relative contribution of each of the different pathways. According to Smith (1985) Put is synthesized mainly from arginine. However, Altman et al. (1982) and Kaur-Sawhney et al. (1982b) suggested that Put is synthesized mainly from ornithine. The existence of a third pathway for polyamine synthesis has also been proposed by Felix and Harr (1989). The nature of this pathway, however, remains unclear.

In senescing carnation flowers Put levels were found to increase sharply simultaneously with the climacteric rise in ethylene production, while the levels of Spd and Spm remained unchanged (Downs and Lovell, 1986; Roberts et al., 1984). These observations indicate that the production of Put during the final stages of senescence does not affect ethylene production. Roberts et al. (1984) suggested that some interaction may exist between the pathways for ethylene and polyamine biosynthesis in which changes in the activity of the polyamine biosynthetic pathway could control the levels of SAM available for ethylene synthesis. Inhibition of polyamine synthesis results in a stimulation of ethylene synthesis (Roberts et al., 1984), while
inhibition of the ethylene pathway results in an increase in Spd levels (Even-Chen et al., 1982; Fuhrer et al., 1982). According to Downs and Lovell (1986) this increase in Spd is caused by an increased channeling of SAM into polyamine biosynthesis. However, evidence exists which indicate that polyamines may inhibit ethylene production mainly through their inhibition of the conversion of ACC to ethylene due to their stabilizing effect on cell membranes and not through their regulatory role on SAM availability (Ben-Arie et al., 1982; Fuhrer et al., 1982; Saftner, 1989). Furthermore, polyamines appear to have very little affect on ACC synthase activity in vivo (Miyazaki and Yang, 1987b).

In *Petunia hybrida* flowers, pollination results in corolla wilting and blueing as well as an acceleration in the increase in ethylene production and sensitivity (Lovell et al., 1987b; Whitehead et al., 1984a; Whitehead and Halevy, 1989). Similar changes were observed in unpollinated flowers, but in this case these changes occurred over a much longer period of time (Whitehead et al., 1984a). *Cyclamen persicum* flowers, on the other hand, produce very little ethylene during senescence and even aged flowers do not react upon treatment with ethylene (Halevy et al., 1984). However, pollination of *Cyclamen* flowers results
in an increase in ethylene production and a stimulation of ethylene sensitivity in the corolla. In this part of the study changes in polyamine levels were monitored in both pollinated and unpollinated flowers to determine the possible relationships between the control of flower senescence and changes in polyamine levels in ethylene sensitive and insensitive flowers.

2.2 MATERIALS AND METHODS

2.2.1 Plant Material

Petunia hybrida (cv. pink cascade) plants were grown in a greenhouse at 28/18°C day/night temperatures under normal seasonal day and night periods. Cyclamen persicum plants were grown under similar conditions except that the day/night temperatures were lowered to 18/15°C to stimulate flowering. All flowers were harvested on the day of their opening and placed in controlled cabinets under continuous illumination from cool white fluorescent lamps (1.5 Wm⁻² PAR) at 22 ±1°C. Flowers were held in distilled water or in the solutions indicated below. Pollination was carried out as described by Whitehead et al. (1984a) and Halevy et al. (1984).
2.2.2 Treatment with Polyamine Inhibitors

In all studies where inhibitors of polyamine synthesis were employed, individual flowers (pollinated or unpollinated) were held in 20ml glass vials containing 10μM or 100μM D-arginine or MGBGH to inhibit Put or Spd and Spm synthesis respectively. Samples were taken from the corollas of these flowers at different time intervals (see Results) and analyzed for their Put, Spd and Spm content by HPLC. Control flowers were held in distilled water for the duration of the experiment.

2.2.3 Effects of Short-Chain Saturated Fatty Acids.

The effect of short-chain saturated fatty acids on the polyamine content of the corolla was examined by applying 2μl droplets of an aqueous solution containing 160ng octanoic acid to the stigmas of freshly harvested unpollinated flowers (3x at 30 min intervals). The octanoic acid solution was prepared by dissolving 160mg of octanoic acid in 1ml pure ethanol. This solution was then diluted to the required concentration with distilled water.
2.2.4 Extraction and Benzoylation of Polyamines.

Polyamines were extracted from corolla tissue according to the method of Flores and Galston (1982) and benzoylated as described by Redmond and Tseng (1979). Samples of corolla tissue (0.5 g) were homogenized with 5 ml of ice-cold 5% (v/v) perchloric acid. After extraction for 1 h on an ice bath cellular debris was removed by centrifugation at 25,000 x g for 20 min at 2°C. To 1 ml of the acidic supernatant 1 ml of 2N NaOH and 5 μl of benzoylchloride were added. The mixture was vortexed for 10 sec and left to stand at room temperature. After 20 min 2 ml of a saturated NaCl solution and 2 ml of diethyl ether were added and the mixture vortexed and centrifuged at full speed in a benchtop centrifuge for 10 min. The ether phase was then removed and evaporated to dryness under nitrogen. The residue was redissolved in 100 μl of methanol and clarified by centrifugation in a microcentrifuge for 5 min before analyzing 25 μl aliquots on a Beckman System Gold HPLC. 1,6-Hexanediamine (HDA) (final concentration 0.1 mM) was added as an internal standard to each sample.
2.2.5 HPLC Analysis.

The derivitized extracts were separated at 25°C on a 250 x 4,6mm Ultrasphere-ODS column (Beckman) using a mobile phase of Methanol : Water (64:36, v/v) at a flow rate of 1ml/min. Peaks were measured at 254nm in channel A of a Beckman Model 167 Scanning Detector. Each peak was scanned from 200 to 350nm in channel B of the detector and the UV spectra (1st, 2nd and log derivatives) were compared with that of authentic polyamines (Put, Spd, and Spm).

2.3 RESULTS

Senescence of unpollinated flowers was accompanied by a decline in the levels of both Put and Spd during the early stages (Fig. 2). Spm was absent from the tissue or present in trace amounts only. The rapid decline in Put during the first day after flower opening was followed by a more gradual decline until the third day. Thereafter the Put levels stayed fairly constant until day 6 when it started to increase again rapidly during the final stages of senescence. The decline in Spd levels followed that of Put. As was the case with Put, Spd also reached a minimum level on the third day after
flower opening after which it stayed fairly constant until day 7 when a further decline was observed (Fig. 2).

In flowers treated with D-arginine, an inhibitor of Put synthesis, Put decreased rapidly to a minimum value during the first day after opening (Fig. 3). This decline was followed by a gradual increase in Put levels until a maximum was reached on the day when the flowers had wilted completely. The levels of Put at this stage were higher than at the time of flower opening. Contrary to the control flowers, the decline in Spd did not follow that of Put, but occurred simultaneously with the decrease in Put levels. The final increase in Spd from day 5 to day 6 followed the increase in Put which occurred from day 2 onward. Treatment with MGBGH, an inhibitor of Spd synthesis, also resulted in a simultaneous initial decrease in the levels in both Put and Spd. However, the Spd levels remained low until the flowers were completely wilted, while the Put levels increase dramatically after day 4 (Fig. 3).

Changes in polyamine levels followed similar patterns in pollinated flowers (Fig. 4) than in unpollinated flowers (Fig. 2). However, in pollinated flowers the process was accelerated so that the decline in Spd
levels did not follow the decline in Put levels, but occurred simultaneously with that of Put (Fig. 4). The Put levels continued to decline during the first 36h following pollination, after which a drastic increase in Put was observed until the final levels exceeded those measured immediately prior to pollination. Spd levels were also at its lowest 36h after pollination. As in unpollinated flowers, Spd remained low until the flowers were completely wilted (Fig. 4). Treatment of pollinated Petunia flowers with D-arginine resulted in an overall decrease in the Put content of the corolla during the first 24h, followed by an increase after 48h. The Spd levels followed the same pattern, but declined again after 60h (Fig. 5). Although treatment of pollinated flowers with MGBGH resulted in a rapid decline in the levels of both Put and Spd during the first 6h after pollination, no definite relationship existed between changes in the levels of Put and Spd. The final irreversible increase in Put was not accompanied by a similar increase in Spd (Fig. 5).

Treatment of unpollinated flowers with octanoic acid did not result in changes in the levels of Put and Spd similar to that observed in pollinated flowers. A gradual overall decline in the levels of both Put and Spd was observed during the first 60h following pollination (Fig. 6). After 60h the Put levels rose to
more than 30μg per gram fresh mass, followed by a final decline during the last 12 hours of the experiment. The Spd levels continued to decline up to 72h after pollination, after which a slight increase was detected during the final 12h period.

In unpollinated Cyclamen flowers senescence was accompanied by an increase in the levels of Put, Spd and Spm during the first three days after flower opening. This rise in polyamine levels was followed by a decrease until day 5. Thereafter no drastic changes were detected in the levels of any of the polyamines until day 15 when Spd started to decline rapidly while Spm increased to a maximum level at day 19. These changes were followed by a rapid decline in Spm during the final stages of senescence. The Put levels remained fairly constant throughout this period (Fig. 7).

Pollination of Cyclamen flowers resulted in similar changes in the levels of all three polyamines during the first 6 days (Fig. 8). Thereafter, the levels of Put and Spd decreased while that of Spm increased until corolla abscission occurred on the seventh day after pollination.
2.4 DISCUSSION

Polyamines play an important role in regulating plant growth and development through their stabilizing effect on cell membranes. Due to their polycationic nature these compounds are able to bind to the negatively charged headgroups of membrane phospholipids (Slocum et al., 1984). This interaction between polyamines and membrane lipids causes a stabilization of the membranes, preventing the senescence-related increase in membrane microviscosity resulting in the maintenance of membrane-related processes such as ion movement and solute uptake (Ben-Arie et al., 1982; De Agazio et al., 1988). Polyamines are also involved in the control of ethylene synthesis via their control of SAM levels in the tissue (Roberts et al., 1984) or through their inhibition of ethylene production from ACC due to their binding to cellular membranes (Saftner, 1989).

It appears that polyamines may play an important role in controlling plant senescence (Kaur-Sawhney et al., 1982a). This effect on senescence processes appears to be very selective, since polyamines are able to inhibit certain aspects of senescence without having an effect on other processes (Suttle, 1981). Polyamines are often implicated as anti-senescence agents, since they are
able to inhibit fruit ripening and leaf senescence (Slocum et al., 1984; Winer and Apelbaum, 1986). Furthermore, in carnations inhibition of polyamine synthesis results in an acceleration of flower senescence (Roberts et al., 1984). According to Slocum et al. (1984) the effect of polyamines in delaying senescence is primarily related to their ability to inhibit ethylene synthesis due to their binding to cell membranes.

Slocum et al. (1984) suggested that the natural onset of senescence is the result of a decrease in the levels of endogenous polyamines. During fruit development polyamine concentrations are at a maximum during the early stages but decrease rapidly to low levels during fruit ripening (Kushad et al., 1988). However, in senescing carnation flowers it was found that the endogenous concentrations of Spd and Spm did not change as flowers aged, but Put increased dramatically and paralleled a sharp rise in ethylene production (Roberts et al., 1984). In Petunia hybrida, flower senescence was accompanied by a rapid decline in the levels of Put and Spd during the pre-climacteric stage. The decline in Spd followed that of Put, indicating that the availability of Put may become limiting in the biosynthesis of Spd during this stage. The rapid rise in Put after the climacteric peak in ethylene
production was reached (Fig. 9) was not accompanied by a similar rise in Spd. This indicates that the tissue had lost the ability to convert Put to Spd or that the availability of SAM was limiting and that Spd synthesis had been inhibited due to a lack of aminopropyl groups derived from SAM (see Fig. 1). The increase in Put during this stage could be the result of a renewed de novo synthesis or the hydrolysis of Put conjugates present in the corolla. Inhibition of polyamine synthesis resulted in an acceleration of flower senescence similar to that observed in carnations by Roberts et al. (1984). Treatment with D-arginine (an inhibitor of the conversion of L-arginine to Put) resulted in an accelerated decrease in the levels of Put, indicating that Put is synthesized from L-arginine via the activity of ADC during the pre-climacteric phase. The increase in Put levels during the later stages of senescence in the presence of D-arginine indicates a change in the pathway of Put synthesis. During this stage Put could be synthesized from ornithine via the activity of ODC or it could be liberated after hydrolysis of Put conjugates in the tissue. The decline in Spd levels during the pre-climacteric phase after treatment with D-arginine appears to be the result of reduced availability of Put. Inhibition of Spd synthesis by treatment with MGBGH resulted in a dramatic increase in
Put levels during the later stages of senescence, possibly due to a reduction in demand for Put used for the synthesis of Spd.

Pollination results in an acceleration of corolla senescence in Petunia flowers (Whitehead et al., 1984a). This acceleration of senescence was accompanied by a rapid decline in the levels of both Put and Spd during the initial stages. As was the case in unpollinated flowers, the increase in Put levels after 36h was not accompanied by an increase in Spd and could not be completely counteracted by treatment with D-arginine. However, the increase in Put was reduced significantly when compared to untreated pollinated flowers, indicating that ADC was still operative at this stage and that Put was still synthesized in part from L-arginine. Treatment with MGBGH did not have a significant effect on the pattern of Put synthesis when compared with control flowers. However, the levels of Spd were not suppressed by treatment with MGBGH as was the case in unpollinated flowers.

Application of short-chain saturated fatty acids to the stigmas of unpollinated Petunia flowers results in a rapid increase in their sensitivity to ethylene and an acceleration in the rate of their senescence similar to that observed in pollinated flowers (Whitehead and
Halevy, 1989). However, application of octanoic acid to the stigmas of unpollinated flowers did not result in a decrease in polyamine levels similar to that of pollinated flowers. In fatty acid treated flowers, senescence was accompanied by a gradual decline in the levels of both Put and Spd up to 60h, indicating that fatty acids do not act by decreasing the levels of polyamines in the corolla.

The flowers of Cyclamen persicum are insensitive to ethylene and senescence is not accompanied by an increase in ethylene production (Halevy et al., 1984). In these flowers, the levels of Put, Spd and Spm increased slightly during the first few days after flower opening. Thereafter it remained fairly constant until the final stages of senescence were reached when a decline in Spd and Spm was observed. Pollination of Cyclamen flowers result in an increase in ethylene synthesis and sensitivity (Halevy et al., 1984). However, pollination-induced senescence was not accompanied by the rapid decrease in polyamine levels observed in pollinated Petunia flowers. In Cyclamen, pollination-induced senescence was characterized by a slight increase in polyamine levels in the corolla similar to that detected in unpollinated flowers. The levels of Spm were, however, lower than in unpollinated flowers. The levels of Spm increased sharply just prior
to abscission of the corolla, while Spd showed a decline during this period. Abscission was followed by a rapid decline in the levels of both Spd and Spm in the corolla. Unlike in Petunia flowers, Put did not increase during the final stages of senescence. The differences in polyamine patterns observed in Petunia and Cyclamen could possibly be ascribed to the differences in their mechanism of senescence. In Petunia senescence is characterized by an increase in ethylene sensitivity and synthesis, while Cyclamen flowers produce little ethylene and remain insensitive to ethylene unless they are pollinated (Halevy et al., 1984; Whitehead et al., 1984a). However, it appears that in both cases senescence is accompanied by a decrease in the levels of the polyamines Spd and Spm during the final stages.

The results of this investigation indicate that polyamines could be actively involved in regulating the onset of senescence in ethylene sensitive flowers such as Petunia, since their levels decrease rapidly during the early stages of senescence in both pollinated and unpollinated flowers. However, in ethylene insensitive flowers such as Cyclamen, the decrease in polyamines appears to be a consequence of senescence which occurs during the final stages.
Figure 2: Polyamines in the corollas of unpollinated senescing Petunia hybrida flowers. (○ Put; ● Spd). Means of 3 flowers.
Figure 3: Polyamines in the corollas of unpollinated senescing *Petunia hybrida* flowers treated with 100µM solutions of D-arg and MGBGH (○ Spd when treated with MGBGH; ■ Spd when treated with D-arg). Means of 3 flowers per treatment.
Figure 4: Polyamines in the corollas of pollinated *Petunia hybrid* flowers (○Put; ●Spd). Means of 3 flowers.
Figure 5: Polyamines in the corollas of pollinated Petunia hybrida flowers treated with 100μM solutions of D-arg and MGBGH (○ Put when treated with MGBGH; ● Put when treated with D-arg; □ Spd when treated with MGBGH; ■ Spd when treated with D-arg). Means of 3 flowers.
Figure 7: Polyamines in the corollas of unpollinated *Cyclamen persicum* flowers (● Put; ■ Spd; ▲ Spm). Means of 3 flowers.
Figure 8: Polyamines in the corollas of pollinated Cyclamen persicum flowers (○ Put; ■ Spd; ▲ Spm). Arrows indicate time of corolla abscission. Means of 3 flowers.
3.1 INTRODUCTION

Senescence of climacteric tissues is characterized by a climacteric rise in ethylene production during the final stages (Burg and Burg, 1965; Sisler and Yang, 1984). The production of ethylene in these tissues is controlled by the activity of ACC synthase, the enzyme responsible for the conversion of SAM to ACC (Adams and Yang, 1979; Cameron et al., 1979; Yu et al., 1979a). In climacteric flowers such as carnation and Petunia, ethylene production is low during the pre-climacteric phase (Nichols et al., 1983; Whitehead et al., 1984a,b). During this phase ACC concentrations are low, but increase simultaneous with the increase in ethylene production (Nichols et al., 1983).

Ethylene is synthesized from the amino acid methionine via SAM and ACC (Adams and Yang, 1979). Since ethylene and polyamines share SAM as a common precursor, it was suggested that senescence could be regulated by the competition between these pathways for SAM (Even-Chen
et al., 1982; Katoh et al., 1987; Roberts et al., 1984). Polyamines may also inhibit ethylene production by inhibiting the conversion of ACC to ethylene (Apelbaum et al., 1981a; Saftner, 1989; Smith, 1985). Such an inhibition of ethylene synthesis would retard senescence in ethylene sensitive tissues.

Senescence of *Petunia hybrida* flowers is accompanied by an increase in ethylene sensitivity which precedes the climacteric rise in ethylene production (Whitehead et al., 1984a). Pollination accelerates this increase in ethylene sensitivity as well as the onset of the rise in ethylene production. It appears that ACC produced in the styles due to the "wounding" of the stylar tissue by the growing pollen-tubes, is responsible for the stimulation of ethylene production in the corollas of pollinated *Petunia* flowers. However, the production of ACC and stimulation of ethylene production is independant from the stimulation of ethylene sensitivity. Ethylene sensitivity is induced by the production of a "sensitivity factor" in the style which is transmitted to the corolla. It appears that short-chain saturated fatty acids may be responsible for the increase in ethylene sensitivity and may act as the "sensitivity factor" in both pollinated and unpollinated flowers (Whitehead and Halevy, 1989). In pollinated flowers the levels of these fatty acids in
the corolla reach a peak within the first 12h after pollination, while in unpollinated flowers the levels of fatty acids increase during the early stages of senescence to reach a peak on the third day after flower opening. In both cases ethylene sensitivity increases simultaneously with the increase in fatty acid concentration. Although the exact mechanism of fatty acid action is still unknown, it is possible that fatty acids may increase ethylene sensitivity by increasing membrane permeability in the tissue (Jackson and Taylor, 1970; Stewart and Berrie, 1979). Cell membranes could also be the site of polyamine action due to their interaction with the negatively charged headgroups of membrane phospholipids.

In this part of the study the effect of polyamines and short-chain saturated fatty acids on ethylene synthesis and sensitivity, as well as membrane permeability were investigated to determine the possible interactions between polyamine and fatty acids and to clarify the mechanisms by which fatty acids may act to increase the sensitivity of the corolla to ethylene.
3.2 MATERIALS AND METHODS

3.2.1 Plant Material

Flowers of *Petunia hybrida* (cv. pink cascade) were obtained from the plants grown in the greenhouse as described in section 2.2.1. Flowers were harvested on the day of opening and held in the growth cabinets as described previously. Pollination was carried out as described by Whitehead *et al.* (1984a).

3.2.2 Ethylene Determination

Ethylene production by *Petunia* flowers was measured by dissecting a single lobe from the corolla of an intact flower at the time intervals indicated in the results. Each lobe was weighed and placed in an 8ml glass tube which was then flushed with ethylene free air and sealed with a rubber septum. The ethylene concentration in the tube was measured after 30 min by withdrawing a 1ml gas sample from the tube and analyzing it on a gaschromatograph equipped with a flame ionization detector and a 1 meter copper column packed with 80 - 100 mesh alumina. Helium was used as carrier gas and the oven temperature was maintained at 100°C. Ethylene
production was calculated as nl ethylene per 1 gram tissue per hour.

3.2.3 Effects of Polyamines and Inhibitors of Polyamine Synthesis on Ethylene Production

To determine the effect of polyamines and inhibitors of polyamine synthesis on ethylene production, flowers were placed in glass vials containing 10 or 100μM solutions of Put, Spd, D-arginine or MGBGH. All flowers were kept in the growth cabinets at 22 ±1°C for the duration of the experiment. Samples were taken at regular intervals and analyzed for their ethylene production as described above.

3.2.4 Effect of Short-Chain Saturated Fatty Acids on Ethylene Production

Unpollinated flowers were treated with octanoic acid and analyzed for ethylene production as described in sections 2.2.3 and 3.2.2.
3.2.5 Measurement of Ethylene Sensitivity

Changes in the sensitivity of pollinated and unpollinated flowers to ethylene were examined by placing harvested flowers in glass vials containing distilled water or 10μM solutions of Put, Spd, Spm, D-arginine or MGBGH. The flowers were then kept in glass chambers at constant temperature (22 ±2°C) and light intensities (1.5 Wm⁻² from cool white fluorescent lamps) and exposed continuously to an air stream containing 4μl ethylene per liter. Control flowers were kept under similar conditions in ethylene free air. The progress of senescence was monitored by assessing changes in the colour of the corollas from pink to blue. Colour changes were scored by ascribing a numerical value to each individual flower corresponding to 0 – all pink; 1 – more pink than blue; 2 – 50% blue; 3 – more blue than pink; 4 – all blue (see plate). A mean colour score was calculated for each set of 5 flowers.

3.2.6 Measurement of Ion Leakage

Changes in membrane permeability were assessed by measuring ion leakage from 8mm diameter discs punched from the upper part of Petunia corollas. In each
experiment discs were punched from ten different 3 day old flowers and floated on a 0.5M mannitol solution for 10 min prior to treatment with polyamines, inhibitors or short-chain saturated fatty acids. Two methods of treatment were employed to examine the effect of the different compounds on membrane permeability. In the first method sets of 10 discs each were floated on different solutions containing 50μM octanoic acid or 10μM Put, Spd or Spm in 0.5M mannitol for 3 hours at 25°C. After incubation in the different solutions, the discs were transferred to clean mannitol solutions and floated for another 10 min period before they were finally placed on 15ml 0.5M mannitol in a clean glass beaker. Each set was then incubated at 25°C and the conductivity of the mannitol solution was measured at hourly intervals during a 5 hour incubation period. In the second method sets of 10 discs each were infiltrated under slight vacuum with 0.5M mannitol solutions containing 10μM Put, Spd or Spm. After infiltration the discs were floated on 0.5M mannitol for 10 min. Discs were then rinsed briefly with double glass distilled water, placed on 15ml 0.5M mannitol and incubated in a shaking water bath at 25°C. The conductivity of the incubation medium was measured at hourly intervals for a 5 hour period.
3.3 RESULTS

Senescence of *Petunia hybrida* flowers was accompanied by a climacteric rise in ethylene production during the final stages (Fig. 9). The maximum in ethylene production was reached on the 6th day after flower opening. Treatment with Put and Spd (10 and 100µM) resulted in an acceleration in the peak of ethylene production by one day and an increase in ethylene evolution during the climacteric stage. The only exception was found in flowers treated with a 100µM Spd solution where the peak in ethylene production was suppressed when compared to untreated flowers. Treatment with inhibitors of polyamine synthesis had a similar effect on ethylene production than treatment with polyamines, except in the case of flowers treated with a 100µM solution of MGBGH where the climacteric rise in ethylene production was accelerated drastically (Fig. 10).

Pollination of *Petunia* flowers resulted in a dramatic acceleration of the climacteric rise in ethylene production and an increase in the amount of ethylene produced when compared with unpollinated flowers (Fig. 11). In these flowers treatment with 10 and 100µM Put and Spd delayed the climacteric peak in ethylene
production except for 10µM Put where the peak in ethylene production coincided with that of the control. However, in all cases ethylene production was higher during the pre-climacteric stage when treated with Put and Spd. Ethylene production was also stimulated during the climacteric phase except in the case of flowers treated with a 100µM Spm where the peak in ethylene production was suppressed. Treatment of pollinated flowers with D-arginine and MGBGH did not affect the timing of the peak in ethylene production, except when treated with a 100µM MGBGH where the peak was accelerated by approximately 12 hours (Fig. 12). Ethylene production was higher in all cases where flowers were treated with the inhibitors of polyamine synthesis.

Application of octanoic acid to the stigmas of unpollinated flowers resulted in an acceleration in the timing of the climacteric peak in ethylene production similar to that observed in pollinated flowers (Fig. 13). However, the levels of ethylene produced during the climacteric stage did not increase when compared to untreated flowers.

Treatment of unpollinated Petunia flowers with 10µM Put and Spd did not affect the rate of senescence as indicated by the rate of corolla blueing (Table 1).
Senescence was, however, accelerated in flowers treated with 10μM solutions of D-arginine or MGBGH. The results from Table 1 indicate that MGBGH was most effective in accelerating senescence in these flowers. In pollinated flowers treatment with 10μM solutions of Put, Spd, D-arginine or MGBGH did not result in a delay in the overall rate of corolla senescence (Table 2). In unpollinated flowers treatment with polyamines resulted in a decrease in ethylene sensitivity when compared to untreated flowers (Table 4). However, inhibition of polyamine synthesis did not affect ethylene sensitivity in these flowers (Table 5). None of these compounds had any effect on ethylene sensitivity in pollinated flowers (Tables 6 and 7).

Application of octanoic acid resulted in a marked acceleration in the rate of corolla blueing when compared to untreated flowers (Table 3). The rate of senescence in fatty acid treated flowers, however, was slightly slower than in pollinated flowers (Tables 2 and 3).

Treatment of isolated corolla discs by floating on octanoic acid and Spm solutions resulted in an increase in electrolite leakage during the 5 hour incubation period, while Put and Spd had little effect. However, vacuum infiltration of Petunia corolla
discs with Put, Spd and Spm resulted in a marked suppression of electrolite leakage from the tissue when compared to discs infiltrated with a pure 0.5M mannitol solution (Fig. 15).

3.4 DISCUSSION

The production of polyamines is a requirement for normal plant growth and development and they may play an important role in the control of plant senescence (Smith, 1985). Polyamines may act as anti-senescent agents by inhibiting ethylene production in ethylene sensitive tissues (Apelbaum et al., 1981b; Ben-Arie et al., 1982; Even-Chen et al., 1982; Suttle, 1981). This inhibition of ethylene production may be caused by a competition between the pathways for polyamine and ethylene synthesis for their common precursor SAM (Even-Chen et al., 1982; Katoh et al., 1987; Roberts et al., 1984) or by the inhibition of the conversion of ACC to ethylene due to their stabilizing effect on cell membranes (Ben-Arie et al., 1982; Fuhrer et al., 1982; Saftner, 1989; Suttle, 1981). According to Saftner (1989) treatment of mature green tomatoes in the pre-climacteric stage with high concentrations of polyamines resulted in an inhibition of ethylene synthesis. However, in senescing Petunia flowers ethylene synthesis was stimulated by treatment with Put
at 10 and 100µM and Spd at 10µM. Treatment with Spd at a 100µM resulted in a suppression of ethylene synthesis, but did not affect the acceleration in the climacteric peak in ethylene production. A similar stimulation of ethylene was observed in Petunia flowers treated with inhibitors of Put and Spd synthesis, confirming the observation made by Roberts et al. (1984) on the effect of polyamine inhibitors in senescing carnation flowers. It is possible that polyamines may regulate ethylene synthesis by a mechanism of feedback control in which the ethylene and polyamine pathways compete for SAM. This would explain the stimulation of ethylene synthesis after inhibition of the polyamine pathway by D-arginine and MGBGH. Furthermore, such a feedback control mechanism could also explain the increase in ethylene production after treatment with polyamines. In this case the increase in endogenous polyamine levels due to polyamine treatment could result in a feedback inhibition of polyamine synthesis resulting in a stimulation of ethylene synthesis. However, in pollinated flowers treatment with Put and Spd resulted in a delay in the climacteric rise in ethylene production, although the levels of ethylene produced in treated flowers were in most cases higher than in untreated flowers. This observation indicates that polyamines did not regulate ethylene synthesis by a feedback control mechanism. Furthermore,
treatment with polyamine inhibitors did not accelerate ethylene production in pollinated flowers, indicating that the availability of SAM is probably not a limiting factor in the synthesis of ethylene. However, competition for SAM by the ethylene and polyamine pathways could provide some degree of regulation of ethylene synthesis, since treatment with polyamines and inhibition of polyamine synthesis results in an increase in the levels of ethylene produced during the climacteric peak.

Although polyamines may play some role in regulating ethylene synthesis, it appears that their effect on decreasing ethylene sensitivity in unpollinated flowers may be more important in the control of flower senescence. This statement is illustrated by the observation that treatment with polyamines did not accelerate the senescence of unpollinated flowers despite the acceleration of ethylene synthesis. Moreover, the acceleration of the onset in the rise of ethylene production by treatment with inhibitors of polyamine synthesis was accompanied by an acceleration in the rate of their senescence. Further evidence in support in this statement is found in the reaction of unpollinated flowers to exogenously applied ethylene. Application of ethylene to unpollinated flowers resulted in a drastic acceleration of their senescence.
However, treatment with polyamines resulted in a delay in the reaction of the flowers to ethylene, indicating that polyamines may act by decreasing the sensitivity of the flowers to this hormone. Treatment of flowers with inhibitors of polyamine synthesis did not affect their ethylene sensitivity. This inability of the inhibitors to increase ethylene sensitivity could be ascribed to the fact that a natural decrease in polyamine levels occurred during the early stages of senescence. In pollinated flowers ethylene sensitivity was not affected by treatment with polyamines or inhibitors, possibly due to the fact that pollination results in a rapid increase in ethylene sensitivity during the first few hours (Whitehead and Halevy, 1989). It appears that the rapid increase in ethylene sensitivity could override the effect of polyamines in decreasing ethylene sensitivity in these flowers.

Application of the short-chain saturated fatty acid octanoic acid, to the stigmas of unpollinated flowers resulted in an acceleration in the rate of their senescence. Although the peak in ethylene production was accelerated similarly to that observed in pollinated flowers, treatment with octanoic acid did not increase the levels of ethylene production during this period as was the case with pollinated flowers. It appears that short-chain saturated fatty acids act
primarily by increasing ethylene sensitivity in the corolla (Whitehead and Halevy, 1989) and not through increasing ethylene production.

The effect of polyamines in delaying plant senescence could be ascribed to their ability to stabilize cellular membranes. In Beta vulgaris it was found that polyamines inhibit solute leakage which resulted from high temperatures, freezing injury and wound-induced destabilization of cell membranes (Altman, 1982b). Polyamines are also able to prevent the natural increase in membrane microviscosity which accompanies senescence (Ben-Arie et al., 1982; Roberts et al., 1986), thus preventing an increase in membrane permeability and ion leakage from the cell (Naik and Srivastava, 1978; Shevyakova, 1982; Srivastava and Smith, 1982). It appears that polyamines may inhibit the senescence-induced destabilization of cell membranes through their polycationic nature which enables them to bind to the negatively charged headgroups of membrane phospholipids. This stabilizing effect of polyamines was also observed in senescing Petunia flowers where treatment with polyamines resulted in a drastic suppression of solute leakage from the tissue. Since ethylene is able to stimulate an increase in membrane permeability (Suttle and Kende,
1980), it appears that polyamines may act by decreasing ethylene sensitivity through their stabilizing effect on cell membranes.

Contrary to the effect of polyamines in decreasing solute leakage from Petunia corolla tissue, treatment with short-chain saturated fatty acids resulted in an increase in solute leakage from the tissue. This observation confirms those of other researchers who found that short-chain fatty acids may cause changes in membrane fluidity resulting in a modification of the activities of exchange systems and an increase in membrane permeability (Berrie, 1979; Berrie et al., 1975; Jackson and Taylor, 1970; Stewart and Berrie, 1979). According to Whitehead and Halevy (1989) treatment of unpollinated Petunia flowers with short-chain saturated fatty acids results in an increase in the sensitivity of the corolla to ethylene similar to that observed in pollinated flowers. Furthermore, treatment of green bananas with fatty acids results in an increase in ethylene sensitivity and ethylene binding (Bosse and Whitehead, unpublished results). It appears that fatty acids may act by increasing membrane permeability which in turn results in an increase in ethylene sensitivity by increasing the ability of ethylene to bind to its membrane-associated binding sites in the cell. It is
possible that this effect of fatty acids on increasing membrane permeability and ethylene sensitivity could be responsible for the inability of polyamines to inhibit ethylene sensitivity in pollinated Petunia flowers. In these flowers pollination is accompanied by a rapid increase in fatty acid levels in the corolla during the first 12 hours (Whitehead and Halevy, 1989). The rapid accumulation in fatty acids could give rise to an early destabilization of cell membranes which could not be counteracted by treatment with polyamines. This would also in part explain the inability of inhibitors of polyamine synthesis to accelerate senescence in pollinated flowers.
Figure 9: Ethylene production by the corollas of unpollinated Petunia hybrida flowers treated with 10 and 100μM solutions of Put and Spd (▲Control; ○10μM Put; ●100μM Put; □10μM Spd; ■100μM Spd). Means of 3 flowers per treatment.
Figure 10: Ethylene production by the corollas of unpollinated *Petunia hybrida* flowers treated with 10 and 100μM solutions of D-arg and MGBGH (▲ Control; ○ 10μM D-arg; ● 100μM D-arg; □ 10μM MGBGH; ■ 100μM MGBGH). Means of 3 flowers per treatment.
Figure 11: Ethylene production by the corollas of pollinated Petunia hybrida flowers treated with 10 and 100μM solutions of Put and Spd (★Control; ○10μM Put; ●100μM Put; □10μM Spd; ■100μM Spd). Means of 3 flowers per treatment.
Figure 12: Ethylene production by the corollas of pollinated Petunia hybrida flowers treated with 10 and 100μM solutions of D-arg and MGBGH (★ Control; ○ 10μM D-arg; ● 100μM D-arg; □ 10μM MGBGH; ■ 100μM MGBGH). Means of 3 flowers per treatment.
Figure 13: Ethylene production by the corollas of *Petunia hybrida* flowers treated with octanoic acid ( *Control* ; ★ Octanoic acid). Means of 3 flowers per treatment.
Figure 14: Effect of octanoic acid, Put, Spd and Spm on solute leafage from Petunia hybrida corolla discs. Discs were treated by floating on solutions containing the different compounds at concentrations indicated for 3 hours prior to conductivity measurements (★ Control; ★ 50μM C₈; ▲ 10μM Put; ○ 10μM Spd; ■ 10μM Spm). Means of 3 replications.
Conductivity ($\mu$Scm$^{-1}$)

HOURS

0 1 2 3 4 5 6

20 10 2 3 4 5
Figure 15: Effects of treatment with Put, Spd and Spm on solute leakage from Petunia hybrida corolla discs. Discs were infiltrated with the different solutions under vacuum prior to conductivity measurements (★ Control; ○10µM Put; ▲10µM Spd; □10µM Spm). Means of 3 replications.
PLATE: Different Stages of Corolla Colour Changes During Senescence

0 - All pink

I - More pink than blue

II - 50% blue
III - More blue than pink

IV - All blue
TABLE 1: Effect of treatment with 10μM solutions of polyamines and inhibitors of polyamine synthesis on corolla colour change in senescing unpollinated Petunia flowers. Means of five flowers per treatment ± SD.

<table>
<thead>
<tr>
<th>TIME (days)</th>
<th>Control</th>
<th>Put</th>
<th>Spd</th>
<th>D-arg</th>
<th>MGBGH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0,6 ± 0,5</td>
<td>0,6 ± 0,5</td>
<td>0,6 ± 0,5</td>
<td>0,6 ± 0,5</td>
<td>0,6 ± 0,5</td>
</tr>
<tr>
<td>3</td>
<td>1,8 ± 0,4</td>
<td>1,2 ± 0,4</td>
<td>1,8 ± 0,4</td>
<td>1,8 ± 0,4</td>
<td>3,0 ± 0</td>
</tr>
<tr>
<td>4</td>
<td>3,0 ± 0</td>
<td>2,8 ± 0,7</td>
<td>3,4 ± 0,8</td>
<td>3,6 ± 0,5</td>
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<tr>
<td>6</td>
<td>4,0 ± 0</td>
<td>4,0 ± 0</td>
<td>4,0 ± 0</td>
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</tbody>
</table>
**TABLE 2**: Effect of treatment with 10\(\mu\)M solutions of polyamines and inhibitors of polyamine synthesis on corolla colour change in pollinated *Petunia* flowers. Means of five flowers per treatment ± SD.

<table>
<thead>
<tr>
<th>TIME (hours)</th>
<th>Control</th>
<th>Put</th>
<th>Spd</th>
<th>D-arg</th>
<th>MGBGH</th>
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</tr>
<tr>
<td>24</td>
<td>0,6 ± 0,5</td>
<td>0,6 ± 0,5</td>
<td>0,6 ± 0,5</td>
<td>0,6 ± 0,5</td>
<td>0,6 ± 0,5</td>
</tr>
<tr>
<td>36</td>
<td>1,2 ± 0,7</td>
<td>1,2 ± 0,7</td>
<td>1,0 ± 0,6</td>
<td>1,0 ± 0,8</td>
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<tr>
<td>48</td>
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</tr>
<tr>
<td>72</td>
<td>4,0 ± 0</td>
<td>4,0 ± 0</td>
<td>4,0 ± 0</td>
<td>4,0 ± 0</td>
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</tr>
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</table>
**TABLE 3:** Effect of treatment with octanoic acid on colour changes in *Petunia* flowers. Octanoic acid was applied to the stigmas of unpollinated flowers. Means of five flowers ± SD.

<table>
<thead>
<tr>
<th>TIME (hours)</th>
<th>COLOUR SCORE Octanoic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>36</td>
<td>0.4 ± 0.5</td>
</tr>
<tr>
<td>48</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>60</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>72</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>84</td>
<td>2.6 ± 1.0</td>
</tr>
<tr>
<td>96</td>
<td>3.4 ± 0.9</td>
</tr>
<tr>
<td>108</td>
<td>4.0 ± 0.0</td>
</tr>
</tbody>
</table>
TABLE 4: Effect of exposure to 4μl·l⁻¹ ethylene on colour changes in unpollinated Petunia flowers treated with 10μM solutions of polyamines. Means of five flowers ± SD.

<table>
<thead>
<tr>
<th>TIME (hours)</th>
<th>COLOUR SCORE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Put</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
</tr>
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<td>8</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>1,0 ± 0</td>
</tr>
<tr>
<td>28</td>
<td>1,4 ± 0,5</td>
</tr>
<tr>
<td>32</td>
<td>2,4 ± 0,5</td>
</tr>
<tr>
<td>36</td>
<td>3,0 ± 0</td>
</tr>
<tr>
<td>40</td>
<td>3,4 ± 0,4</td>
</tr>
<tr>
<td>48</td>
<td>4,0 ± 0</td>
</tr>
<tr>
<td>52</td>
<td>4,0 ± 0</td>
</tr>
</tbody>
</table>
TABLE 5: Effect of exposure to $4\mu l.l^{-1}$ ethylene on colour changes in unpollinated Petunia flowers treated with $10\mu M$ solutions of polyamine inhibitors. Means of five flowers ± SD.

<table>
<thead>
<tr>
<th>TIME (hours)</th>
<th>COLOUR SCORE</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>D-arg</td>
<td>MGBGH</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>$0.8 \pm 0.4$</td>
<td>$0.5 \pm 0.5$</td>
<td>$0.8 \pm 0.4$</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>$1.0 \pm 0$</td>
<td>$1.0 \pm 0$</td>
<td>$1.3 \pm 0.4$</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>$1.8 \pm 0.4$</td>
<td>$1.5 \pm 0.8$</td>
<td>$1.6 \pm 0.7$</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>$2.8 \pm 0.7$</td>
<td>$2.6 \pm 0.5$</td>
<td>$2.6 \pm 0.5$</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>$3.2 \pm 0.4$</td>
<td>$3.0 \pm 0$</td>
<td>$3.0 \pm 0$</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>$3.6 \pm 0.5$</td>
<td>$3.8 \pm 0.4$</td>
<td>$3.8 \pm 0.4$</td>
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</tr>
<tr>
<td>52</td>
<td>$4.0 \pm 0$</td>
<td>$4.0 \pm 0$</td>
<td>$4.0 \pm 0$</td>
<td></td>
</tr>
</tbody>
</table>
TABLE 6: Effect of exposure to 4μl.l⁻¹ ethylene on colour changes in pollinated Petunia flowers treated with 10μM solutions of polyamines. Means of five flowers ± SD.

<table>
<thead>
<tr>
<th>TIME (hours)</th>
<th>Control</th>
<th>Put</th>
<th>Spd</th>
<th>Spm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>0,6 ± 0,5</td>
<td>0,4 ± 0,5</td>
<td>0,6 ± 0,5</td>
<td>0,6 ± 0,5</td>
</tr>
<tr>
<td>30</td>
<td>0,8 ± 0,4</td>
<td>1,4 ± 0,8</td>
<td>1,6 ± 0,5</td>
<td>1,4 ± 0,8</td>
</tr>
<tr>
<td>36</td>
<td>1,8 ± 0,7</td>
<td>2,4 ± 0,5</td>
<td>2,6 ± 0,5</td>
<td>2,4 ± 0,5</td>
</tr>
<tr>
<td>42</td>
<td>3,0 ± 0</td>
<td>3,0 ± 0</td>
<td>3,6 ± 0,5</td>
<td>3,4 ± 0,8</td>
</tr>
<tr>
<td>48</td>
<td>4,0 ± 0</td>
<td>4,0 ± 0</td>
<td>4,0 ± 0</td>
<td>4,0 ± 0</td>
</tr>
</tbody>
</table>
**TABLE 7**: Effect of exposure to 4μl·l⁻¹ ethylene on colour changes in pollinated *Petunia* flowers treated with 10μM solutions of polyamine inhibitors. Means of five flowers ± SD.

<table>
<thead>
<tr>
<th>TIME (hours)</th>
<th>CONTROL</th>
<th>D-arg</th>
<th>MGBGH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>0,6 ± 0,5</td>
<td>0,8 ± 0,4</td>
<td>0,4 ± 0,5</td>
</tr>
<tr>
<td>30</td>
<td>0,8 ± 0,4</td>
<td>1,0 ± 0</td>
<td>1,2 ± 0,5</td>
</tr>
<tr>
<td>36</td>
<td>1,8 ± 0,7</td>
<td>2,0 ± 0</td>
<td>2,2 ± 0,4</td>
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<tr>
<td>42</td>
<td>3,0 ± 0</td>
<td>3,2 ± 1,0</td>
<td>3,4 ± 0,8</td>
</tr>
<tr>
<td>48</td>
<td>4,0 ± 0</td>
<td>4,0 ± 0</td>
<td>4,0 ± 0</td>
</tr>
</tbody>
</table>
CHAPTER 4

SUMMARY AND CONCLUSION

The results of this study clearly demonstrate that both polyamines and short-chain saturated fatty acids play an important role in regulating senescence in ethylene sensitive tissues such as the corolla of Petunia hybrida flowers. In Petunia flowers Put and Spd appears to be the most abundant polyamines, while Spm was absent or present in trace amounts only. Senescence in these flowers, whether natural or pollination-induced, was accompanied by a rapid decline in the concentrations of Put and Spd during the early stages. It appears that the decline in Spd was the result of a reduced availability of Put. However, the increase in Put during the later stage of senescence was not accompanied by an increase in Spd synthesis, indicating that the tissue had lost the ability to synthesize Spd during this stage. Put is probably synthesized from L-arginine during the early stages of flower senescence, but the rise in Put during the final stages could not be inhibited by D-arginine, indicating that Put was synthesized via an alternative pathway (possibly from ornithine?) or that it was liberated due to the hydrolysis of Put conjugates.
It was suggested by Slocum et al. (1984) that the onset of senescence is the result of a decline in polyamine levels. However, in this study it was found that continuous treatment with polyamines did not delay senescence in *Petunia* flowers, indicating that senescence did not result from a lack of available polyamines. It appears, however, that polyamines may act by decreasing the sensitivity of the tissue to ethylene by stabilizing the cell membranes. This would explain in part the increase in ethylene sensitivity observed by Whitehead and Halevy (1989) during senescence following the decrease in polyamine levels detected in this study. The results of this study indicate that polyamines act primarily by stabilizing cell membranes and decreasing the sensitivity of the tissue to ethylene. Its effect on ethylene synthesis does not appear to play an important role in the control of senescence.

Although polyamines could play an important role in controlling ethylene sensitivity in plant tissues, it seems that other factors may also be involved in this process. In pollinated *Petunia* flowers, treatment with polyamines did not have an effect on their sensitivity to ethylene. According to Whitehead and Halevy (1989) short-chain saturated fatty acids may be responsible for the increase in ethylene sensitivity in both
pollinated and unpollinated flowers. It appears that these fatty acids do not act by inhibiting polyamine synthesis or stimulating ethylene synthesis. Their ability to increase ethylene sensitivity seems to be related to their ability to destabilize cell membranes, resulting in an increase in solute leakage and an increase in the ability of the tissue to bind ethylene. The results of this and other studies indicate that polyamines and short-chain saturated fatty acids may interact to control ethylene sensitivity in plant tissues. In pollinated flowers the levels of fatty acids increased rapidly during the first 12h following pollination (Whitehead and Halevy, 1989). This increase in fatty acid concentrations was accompanied by a simultaneous decline in polyamine levels during the first 36h. The same patterns were also observed in unpollinated flowers where the levels of fatty acids increased during the first three days after flower opening (Whitehead and Halevy, 1989) while the polyamine levels declined during the same period. However, it appears that short-chain saturated fatty acids may be more important than polyamines in regulating ethylene sensitivity in plant tissues, since treatment of unpollinated flowers with fatty acids resulted in a rapid increase in ethylene sensitivity (Whitehead and Halevy, 1989) while having little effect on polyamine synthesis during the early stages of
senescence. Furthermore, polyamines were not able to decrease ethylene sensitivity in pollinated flowers, possibly due to the rapid increase in fatty acid levels in the corolla. In this case the destabilization of cell membranes by fatty acids could possibly override the stabilizing effect of exogenously applied polyamines.

The exact role of polyamines in ethylene insensitive tissues remains unclear. In Cyclamen persicum flowers senescence was accompanied by an initial increase in polyamine levels during the first three days after flower opening. In these flowers the levels of Spd declined only during the final stages of senescence, while the levels of Put and Spm remained low during the whole senescence process. The slow changes in polyamine levels could in part explain why these tissues remained insensitive to ethylene. Although pollination of Cyclamen flowers resulted in an increase in ethylene sensitivity (Halevy et al., 1984), senescence of the flowers was not accompanied by wilting of the corolla. In these flowers senescence is characterized by abscission of the corolla while still fully turgid. An increase in ethylene sensitivity does not relate to the senescence of the whole corolla as in Petunia, but refers to the stimulation of corolla abscission. The abscission process is localized in a few cell layers in
the abscission zone. This may explain why Spd levels remained high until abscission had occurred and why an increase in Spm just prior to abscission did not prevent the process. However, more research needs to be done before the exact role of polyamines in these tissues could be explained.
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