THE PHYSIOLOGICAL RESPONSE OF CUT CARNATION FLOWERS TO ETHANOL AND ACETALDEHYDE POST-HARVEST TREATMENTS

by

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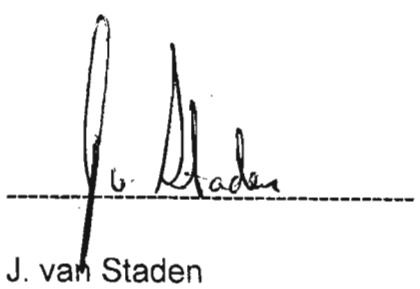
PREFACE

I hereby declare that this thesis, submitted for the degree of Doctor of Philosophy in the faculty of Science and Agriculture, University of Natal, Pietermaritzburg, except where the work of others is acknowledged, is the result of my on investigation.

Lindsey Alice Podd

November, 2000

I certify that the above statement is correct



J. van Staden

Supervisor

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In loving memory of MARGARET KISS and LEONARD PODD

ABSTRACT

A replacement for silver thiosulphate as a commercial post-harvest treatment needs to be found. The longevity of cut carnation flowers is extended by all concentrations of ethanol tested. Compared to a water control, the vase-life of ethanol-treated flowers is between 150 and 250% longer. The greatest longevity increases are recorded with 3% ethanol. The use of ethanol as a post-harvest treatment was tested. The longevity increase as a result of ethanol application only occurs if the ethanol is applied as a holding solution. Pulse treatments are not effective at delaying the senescence of the flowers. The sooner the ethanol is applied, the greater the increase in vase life. If ethanol treatment is halted at any point during the experiment, the longevity of the flowers is reduced. It was observed that the longer the stems of ethanol-treated flowers, the greater the longevity increases. The ethanol holding solution does not prevent the action of external ethylene, thereby restricting the potential of ethanol as a commercial post-harvest treatment.

Physiologically, flowers treated with ethanol exhibit a different senescence process to control flowers. The typical in-rolling of the petals of carnation flowers is not seen, instead the petals appear burnt. The ovaries are also notably effected by ethanol, being smaller and more yellow in colour. Ethanol treatment results in longevity increases by inhibiting the formation of ethylene, the plant hormone responsible for senescence. The concentration of the direct precursor to ethylene, ACC, as well as the activity of the enzyme that converts ACC to ethylene, ACC oxidase, is reduced to almost zero in the tissues of treated flowers. Another

physiological factor affected by ethanol treatment is the carbohydrate status of the flowers. The normal sink activity of the ovary is inhibited by ethanol treatment. Although the carbohydrate content of the petals is found to decrease sharply in ethanol-treated flowers, these carbohydrates are not relocated to the ovary. The ovary does not increase in dry matter or chlorophyll content. The carbohydrate content decreases as a result of ethanol treatment, and when ^{14}C sucrose was applied to petals, no radioactivity was recovered in the ovary. The petals and ovary are the organs most effect by ethanol activity, as when ^{14}C ethanol was applied to cut carnation flowers as a pulse, the majority of the radioactivity was discovered here. The protein content of cells of both organs decreases significantly compared to control flowers. This is a total protein loss, rather than the destruction of specific systems.

If the activity of alcohol dehydrogenase is prevented in ethanol-treated flowers, inhibiting the conversion of ethanol to acetaldehyde, no longevity increases are seen. The airspace surrounding treated flowers was found to contain ethanol and small amounts of acetaldehyde. The tissues of flowers treated with ethanol show an increase in the acetaldehyde content, as well as the ethanol content, especially in the ovary. The application of acetaldehyde directly to cut carnation flowers as a holding solution resulted in the vase life of the flowers increased by 150%.

To determine the effectiveness of acetaldehyde as a post-harvest treatment, various concentrations of acetaldehyde were applied to cut carnation flowers as a pulse treatment and a holding solution. Pulse treatments did not increase the vase

life of flowers, and resulted in a number of negative effects in the flower. A holding solution of acetaldehyde does increase the longevity of cut carnation flowers, provided it is above a certain concentration. Treatments at concentrations below 1% acetaldehyde appear to promote flower senescence. The use of acetaldehyde as a post-harvest treatment has many of the same disadvantages as ethanol treatment. Acetaldehyde must also be applied as a holding solution for as long as possible. If removed from this solution, death of the organ occurred quickly. Acetaldehyde is also ineffective against external ethylene. A negative effect of acetaldehyde not found in ethanol-treated flowers, is that the longer the stem of cut carnation flowers, the shorter the resultant vase life.

Physiologically the responses in cut carnation flowers were very similar to those seen in ethanol-treated flowers. Acetaldehyde inhibited the formation of ethylene completely. Almost no ACC can be found in treated tissues, and the action of ACC oxidase is completely reduced. The petals of acetaldehyde-treated flowers suffer from severe petal browning, rather than in rolling. The ovaries are particularly badly effected by treatment. There are large scale losses in fresh weight and chlorophyll content. The latter results in the ovaries appearing yellow in colour. They also show a loss in structure. The sink activity of these ovaries is destroyed. Like ethanol-treated flowers, the carbohydrate content of both the petals and ovaries are dramatically reduced. When ^{14}C sucrose was applied to one of the petals, almost no radioactivity was recorded in the ovary. There is also a major loss in general protein content, slightly more severe than in ethanol-treated flowers.

The conversion of ethanol to acetaldehyde is necessary in order to achieve longevity increases in ethanol-treated flowers. If the conversion of this acetaldehyde to ethanol is prevented in acetaldehyde-treated flower there is once again no longevity increase. Both ethanol and acetaldehyde are required within the system to result in increased longevity. Although ethanol and acetaldehyde treatments result in decreases in the total protein content of the flowers, certain enzymes remain active. Alcohol dehydrogenase is a bi-directional enzyme, capable of converting ethanol to acetaldehyde and then back to ethanol again. The activity of this enzyme, in both orientations, is increased in ethanol and acetaldehyde-treated flowers. The activity of pyruvate decarboxylase, which converts pyruvate to acetaldehyde, is also increased as a result of both treatments. The similarities of the physiological response of cut carnation flowers to ethanol and acetaldehyde post-harvest treatments, and the increased activity of these enzymes, indicate that the effect of both compounds on longevity is closely linked.

PUBLICATIONS FROM THIS THESIS

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Ethanol as a post-harvest treatment for cut carnation flowers. Presented at the 23rd annual meeting of the South African Association of Botanists, held in Cape Town in January 1998.

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
2,4-D	2,4-Dichlorophenoxy-acietic acid
2,5-ND	2,5-Norbornadine
ABA	Abscisic acid
AcA	Acetaldehyde
ACC oxidase	1-Aminocyclopropane-1-carboxylic acid oxidase
ACC synthase	1-Aminocyclopropane-1-carboxylic acid synthase
ACC	1-Aminocyclopropane-1-carboxylic acid
ADH	Alcohol dehydrogenase
ANOVA	One way analysis of variance
AOA	Amino-oxyacetic acid
APS	Ammonium persulfate
AVG	Amino-ethoxy vinylglycine
BA	Benzyladenine
*bis*acrylamide	N'N' Methylenebisacrylamide
BSA	Bovine-serum A
$Ca(NO_3)_2$	Calcium nitrate
Chl	Chlorophyll
cm	Centimetre
CO_2	Carbon dioxide
Da	Dalton
ddH_2O	Deionised distilled water
DMF	N,N-Dimethylformamide
DNA	Deoxyribonucleic acid
DPSS	1,1-Dimethyl-4-(phenylsullfonyl)semicarbazide
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EFE	Ethylene forming enzyme
EMP	Embden-Meyerhof-Parnas
Ethrel ®	2-Chloroethyl phosphonic acid
EtOH	Ethanol

g	Gram
g	Gravity
h	Hour
H_2O_2	Hydrogen peroxide
$HgCl_2$	Mercuric chloride
IAA	Indolacetic acid
KDa	Kilodaltons
K_2SO_4	Potassium sulphate
KCl	Potassium chloride
KNO_3	Potassium nitrate
ℓ	Litre
MA	Milliamps
mACC	N-Malonyl-1-aminocyclopropane-1-carboxylic acid
mCi	Microcurrie
mg	Milligram
min	Minute
mℓ	Millilitre
mmol	Millimole
MRNA	Messenger ribonucleic acid
MTA	5'-Methylthioadenosine
MVG	Methoxy vinylglycine
N	Normal
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide phosphate
NaOCl	Sodium hypochlorite
$NaPO_4$	Sodium phosphate
NH_4NO_3	Ammonium nitrate
NH_4OH	Sodium hydroxide
nm	Nanometre
PCl acid	Perchloric acid
PDC	Pyruvate decarboxylase
PMSF	Phenylmethylsulfonyl fluoride
PVPP	Polyvinylpolypyrollidone

Rpm	Revolutions per minute
S100	100% Senescence
S50	50% Senescence
SAM synthetase	S-adenosylmethionine
SAM	S-adenosylmethionine
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis
Sec	second
Std-dev	Standard Deviation
STS	Silver thiosulphate
TCA	Trichloroacetic acid
Temed	NNN'N'Tetramethylethylenenediamine
Tris	2-Amino-2(hydroxymethyl)-propane-1,3-diol
UV	Ultraviolet
µCi	Microcurrie
µg	Microgram
µℓ	Microlitre
µM	Micromole

CHAPTER 1

LITERATURE REVIEW

1.1. Senescence

According to Webster's Dictionary of Scientific Terms (ALLEE, 1980), to be senescent is defined as the "period in the life history of an individual when its powers are declining prior to death", while senescence is "the process of growing old". All organisms experience a period of senescence, from a mighty bull elephant to a humble bacterium. Biologically speaking, senescence is an endogenously controlled, degenerative process that leads to cell death in the organ or organism (LEOPOLD, 1975). Generally occurring at the end of a period of growth and development, senescence of an organ or organism may include:

- preparation for subsequent changes;
- the breakdown process itself; and
- preparation for another period of growth (THIMANN, 1988).

1.1.1. Flower senescence

Regarding senescence as preparation for another period of growth is particularly true for flower senescence. For even as the flower itself is wilting and fading, the development of fruit and seed within the floral head holds the key to the future of the species. Flowers, like all organisms, exhibit an organised pattern of development. Floral induction of a vegetative shoot stimulates the apical meristem to form an assemblage of sterile and reproductive flower parts, enclosed as a bud. It is from this calyx-protected bud that the petals emerge, attracting pollinators to the exposed stigma and stamens

(COOK, 1985). Flowers house the ovary, which is the site of pollination, fertilisation and seed set. As vital as these organs are to the survival of angiosperm species, flowers are however, extremely energy expensive. Therefore, once pollination has occurred, or after a specific period of time even if it has not occurred, organ death begins (GILISSEN, 1977). In monocarpic plants flower senescence heralds the dramatic death of the whole plant. In polycarpic plants, such as the carnation, senescence is restricted to the flower itself, while the rest of the plant survives to the following growing season.

Flowers consist of more morphological parts than any other organ of the plant. Therefore senescence in flowers is more complex than in fruit, leaves or stems. Organs such as the petals, ovary, receptacle and stamens all interact during the senescence process (MAYAK and HALEVY, 1980). If the death of one plant part or organ is influenced by the growth of another, the senescence process is referred to as correlative. Flowers are composed of a diverse number of parts senescing at different rates, and their senescence is therefore considered to be a correlative process. It is this correlative senescence that determines the overall appearance and survival of the flower (SETH and WAREING, 1967; NOODEN, 1988).

1.1.2. Cut flower senescence

Cut flowers are flowers that have been detached from the mother plant. Flowers are already the organs of the plant with the shortest life span, but harvesting a bloom from the parent plant accelerates the overall senescence of this organ even further (MAYAK and HALEVY, 1980). The senescence of cut flowers was originally regarded as being due to the starvation of the excised organ (MÖLISCH, 1928). With the subsequent realisation that this process requires the metabolism of breakdown products,

including the translocation of carbohydrates and nitrogenous compounds into other floral parts, it is now considered a normal stage of growth (COOK, 1985). While attached to the parent plant, losses of energy from the flower due to respiration and transpiration are replenished from the assimilate pool of the parent, as well as water and nutrient uptake by the roots. Removal of the flower from these nutrient sources makes the explant entirely dependent on its own food reserves and moisture content. If these requirements are not supplemented, they become limiting. As soon as this occurs, flower senescence has begun. The rate at which this situation becomes limiting is largely dependant on the kind and intensity of the physiological status of the flower (COOK, 1985). The length of time for which cut flowers are visually acceptable is regarded as their vase-life. The longer it takes for the nutrient and water situation to become limiting within the flower, the longer the vase-life will be. The physical impact of flower senescence is normally judged by the appearance of the petals (NICHOLS, 1977b). The vase-life of flowers was determined to be over when petal senescence had occurred. Petals were considered to be senesced when they had wilted, shrivelled, faded or abscised (MAYAK and DILLEY, 1976a).

The cut flower industry is worth some $60 billion (U.S.) annually, and the longevity of their vase-life is an important factor in consumer preference (PUN, ROWE, ROWARTH, BARNES, DAWSON and HEYES, 1999). A great deal of research has been conducted with the intention of extending the longevity of these commercially important organs. Many chemical methods of increasing the vase-life, and overall longevity, of cut flowers have been developed. Limited use has also been made of natural plant hormones in flower preservation (UPFOLD, 1992).

1.1.3. Carnation flower senescence

Carnations (*Dianthus caryophyllus*) are one of the most popular cut flowers, ranking fifth in the world (VONK NOORDEGRAFF, 1998), and account for $85 million (US), in domestic as well as export sales in the Dutch auction market in 1995 (PUN, ROWE, ROWARTH, BARNES, DAWSON and HEYES, 1999). Of all flowers researched in terms of their post-harvest physiology, none has been used more often than the carnation. This is because carnations are autocatalytic, climacteric flowers, exhibiting a marked burst in ethylene production and respiration. Both of these are easy to monitor, thereby making carnation flowers an ideal model for senescence studies. Carnations are also affordable and available year round, and there are a number of internationally available cultivars (COOK, 1985).

1.2. The physiology of senescing cut carnation flowers

1.2.1. Petal senescence

Petal senescence is the factor that determines the vase-life of the flowers. The senescence of cut carnation petals can be divided into two stages:

- an initial, reversible phase of active proteolysis during which a moderate loss of electrolytes occurs; and
- an irreversible phase, which is preceded by ethylene production and leads to the death of the flower (TRIPPI and PAULIN, 1984).

Reversible petal senescence results in petals appearing limp, and occurs due to losses in tugor, a symptom of water stress. This can be reversed by increasing the amount of water in the system (NICHOLS, 1977b).

Irreversible wilting is connected to ethylene production, and results in the progressive in-rolling of the petals, wilting or colour changes. In carnations the time-course of ethylene production is triphasic, a low steady-state level, followed by a climacteric rise, then a decline. Visual symptoms of petal senescence become evident at the end of the second stage (SMITH, SAKS and VAN STADEN, 1992). These have generally been associated with increased membrane permeability and loss of compartmentation (KENDE and BAUMGARTNER, 1974; EZE, MAYAK, THOMPSON and DUMBROFF, 1986; SMITH, SAKS and VAN STADEN, 1992). Post-climacteric petal tissue is characterised by large-scale ultra-structural disorganisation, including the collapse of the epidermal cells, vacuolar membranes in the mesophyll being damaged, vacuole shrinkage and rupture, and cell wall and plasmalemma rupture (SMITH, SAKS and VAN STADEN, 1992). This irreversible petal damage terminates in the death of the petals, and therefore signifies the end of the vase-life of the flowers (NICHOLS, 1977b). Concurrent with this petal wilting, the ovary "greens", gains weight, and if pollinated, sets seed (COOK, 1985).

1.2.2. Water deficiency

Water deficit in cut flowers is considered to be a major cause of rapid senescence (AARTS, 1957; LEVITT, 1972; ROGERS, 1973). A reduction in the moisture content of the petals will result in wilting of the petals, and thus a reduction in the vase-life. The picking of the flowers initially causes water deficiency within the system. No matter how little time elapses between picking, and placing the flowers in a holding solution, some water stress will occur. Poor post-harvest handling can even result in accelerated senescence (MAYAK and HALEVY, 1974; BOROCHOV, TIROSH and HALEVY, 1976). As well as reducing the availability of water within the system, picking flowers limits their

ability to take up water (HALEVY and MAYAK, 1981). Even if flowers receive the best possible post-harvest handling and are held in water continually, some level of water stress will always take place (MAYAK and HALEVY, 1974). Such stress was originally thought to be due to deposits plugging the xylem vessels, and hence obstructing water uptake (DURKIN and KUC, 1966; LARSEN and FRÖLICH, 1969; RASMUSSEN and CARPENTER, 1974). Xylem blockages arise from the growth of micro-organisms within the stem tissue (LARSEN and CROMARTY, 1967; LARSEN and FRÖLICH, 1969; ROGERS, 1973), as well as the release of products resulting from oxidative processes (DURKIN and KUC, 1966; LINENBERGER and STEPONKUS, 1976). However, if blockage of the xylem tissue is prevented, no significant relationship exists between xylem blockage and flower longevity (CAMPRUBI and FONTARNAU, 1977; COOK, 1985). Apart from problems with water uptake, water stress can also be caused by stomatal behaviour. Stomatal movement is affected by plant hormones. Cytokinins and gibberellins cause stomata to open (LIVINE and VAADIA, 1965). Auxin (FERRI and LEX, 1948), abscisic acid (JONES and MANSFIELD, 1970) and ethylene (MADHAVAN, CHROMINSKI and SMITH, 1983) cause stomatal closure. Ethylene, abscisic acid and auxin also promote flower senescence. This indirectly suggests that continued water flow and gaseous exchange may be an impetus needed to maintain flower vitality (COOK, 1985). These problems of stomatal behaviour and water uptake will result in reduced photosynthesis, disruption of assimilate movement and growth inhibition, including the accumulation of nutrients in storage organs (HODDINOTT, EHRET and GORHAM, 1979; KURSANOV, 1984). Stomatal closure, in particular, will result in reduced gaseous exchange, directly effecting photosynthesis and respiration (COOK, 1985).

1.2.3. Respiration

Senescing carnation flowers do not show a gradual decrease in respiration, as is seen in some other types of flowers, such as roses (KALTALER and STEPONKUS, 1976). Instead there is an initial decline in the respiration rate after cutting, followed by a steep increase to a peak, and then a second decline. This precisely mirrors the respiration pattern seen in climacteric fruit (SOLOMOS, 1988). This climacteric respiration increase coincides almost exactly with the final decline in the life of the flower, especially in terms of petal wilting (NICHOLS, 1968; HALEVY and MAYAK, 1979; COOK, 1985; SOLOMOS, 1988). Increases in the rate of respiration have been attributed to a decrease in organisational resistance (BLACKMAN and PARIJA, 1928), enhancement of protein synthesis (HULME, 1954), the presence of natural uncouplers of oxidative phosphorylation (MILLERD, BONNER and BIALE, 1953), and an attempt by the tissues to maintain metabolic homeostasis (ROMANI, 1984). However, the only common metabolic feature of all climacteric fruit and flowers is their responsiveness to ethylene. During the senescence of climacteric fruit and flowers there is a huge increase in ethylene production. There is also a response to exogenous application of this gaseous hormone by increasing the respiration rate, and promoting eventual senescence. This indicates that the climacteric respiration increase seen in carnation flowers and other climacteric organisms is in fact not an individual senescence response, but rather a facet of ethylene action (SOLOMOS, 1988). Ethylene, in theory, stimulates respiration by inducing the *de novo* synthesis of respiratory enzymes (DILLEY, 1962; HULME, RHODES, GALLIARD and WOOLTORTON, 1968; SALMINEN and YOUNG, 1975). However, although increases in relevant enzymes such as malic enzyme and phosphofructokinase were seen in some fruit, the respiratory potential of preclimacteric tissues is adequate to sustain the climacteric respiratory peak (MILLERD,

BONNER and BIALE, 1953; LATIES, 1978; DAY, ARRON and LATIES, 1980). This suggests that ethylene enhances respiration by activating a pre-existing enzymatic potential (SOLOMOS, 1988).

1.2.4. Carbohydrate status

Of the stored compounds found in plant tissue, more is known about starch and sucrose than all the other storage compounds combined. Glucose, fructose, inositol and xylose are all found in the ovary of senescing carnation flowers, (NICHOLS and HO, 1975a; NICHOLS and HO, 1975b; HALEVY and MAYAK, 1979; DIMALLA and VAN STADEN, 1980; DUCASSE and VAN STADEN, 1981; COOK and VAN STADEN, 1983; COOK, 1985; COOK and VAN STADEN, 1986; COOK and VAN STADEN, 1988). The absence of starch from this list suggests that sucrose is not only the principal translocatory compound, but that it could also be the main storage sugar. This makes sucrose an important constituent of carnation flowers (NICHOLS and HO, 1975a; COOK, 1985). Once a flower has been severed from the parent plant, the major source of carbohydrates has been removed from the system. A sink is a region within a plant that has high metabolic activity, resulting in assimilates being utilised (NICHOLS and HO, 1975a; NICHOLS and HO, 1975b). If a cut flower has been pollinated, the ovary will now act as a sink, requiring all of the mobilised reserves within the flower in order to develop into fruit. In this case, all the other organs will act as a source. A source is a region of production within the flower, which provides metabolites for the sink. When a source-sink situation occurs, assimilates are mobilised from the photosynthetic or storage tissues of the source, and are transported to the sink for metabolism (WARREN-WILSON, 1972; NICHOLS, 1973; NICHOLS and HO, 1975a; NICHOLS and HO, 1975b). Even if pollination has not occurred, in cut carnation flowers, the petals act as

a source that provides carbohydrates to the ovary (DIMALLA and VAN STADEN, 1980). During senescence, the levels of soluble sugars in the petals gradually decline. Stored energy, in the form of carbohydrates, is shunted from the petals to the ovary, via the receptacle, which both organs are attached to. This movement promotes irreversible wilting of the petals, as the osmotic balance of the tissues cannot be maintained without the chemical constituents remaining in the petals (DIMALLA and VAN STADEN, 1980). In order to maintain the ovary as a sink however, a sucrose gradient must exist. The sucrose must move from a region of high sucrose concentration, to a region of low sucrose concentration. Therefore the sucrose in the ovary must either be stored, or metabolised. As there is very little starch present in the ovary, it appears that the sucrose is metabolised (COOK and VAN STADEN, 1986). This requires three enzymes: sucrose synthetase, acid invertase and alkaline invertase. Of these, acid invertase is the most active in the ovary of cut carnation flowers. In fact, the unloading and utilisation of sucrose by the sink correlates directly with the amount of acid invertase activity present in the tissue (SHANNON and DOUGHERTY, 1972). This constant movement of sucrose from the petals to the ovary, via the sucrose gradient, is the determining factor in petal wilting, and therefore flower senescence itself (COOK and VAN STADEN, 1986).

1.3. The role of plant hormones in cut carnation flower senescence

1.3.1. Ethylene

Ethylene is a simple gaseous plant hormone that is produced in almost every cell of the plant. It can be transported from cell to cell by diffusion, the xylem, the phloem and even atmospherically from one plant part to another, or from plant to plant. The

activity of ethylene was first discovered in the 1800's, when streets were still illuminated by means of gas lamps. It was observed that increased defoliation occurred in the trees closest to these lamps. In 1901 DIMITRRY NELJUBOV demonstrated that the active component produced by the lamps was ethylene, which caused pea seedlings to grow horizontally (ABELES, 1973). Ethylene occurs naturally in the atmosphere as a result of industrial pollution and the burning of fossil fuels. In 1910 it was found to occur naturally in plants, as a gas released by oranges that caused bananas to ripen (COUSINS, 1910). It was identified as a plant hormone in 1934 (GANE, 1934). CROCKER and KNIGHT (1908) first reported the sensitivity of flowers to ethylene. Ethylene was the first plant hormone shown to be involved in the final stages of flower senescence (ABELES, 1973; COOK, 1985). In the presence of ethylene, be it endogenous, applied, or atmospheric, the longevity of cut carnation flowers is greatly reduced (COOK and VAN STADEN, 1988). In fact, when compared to other commercially important flowers, carnations are particularly sensitive to ethylene (SMITH, PARKER and FREEMAN, 1966; MAYAK and DILLEY, 1976b). In cut carnations the presence of ethylene signals the onset of termination of flower life. Prior to the irreversible wilting of carnation petals, their rate of ethylene production increased dramatically, and peaked at the onset of petal in-rolling (NICHOLS, 1968; DOWNS and LOVELL, 1986a; COOK and VAN STADEN, 1988). To determine why there was this sudden increase in ethylene production, and how senescence is regulated by it, it is necessary to consider ethylene biosynthesis. The ethylene biosynthetic pathway is relatively simple, only four steps and three enzymes are involved (FIGURE 1.1.)

1.3.1.1. Ethylene biosynthesis

Methionine is the major precursor of ethylene (LIEBERMAN, 1979). It is present

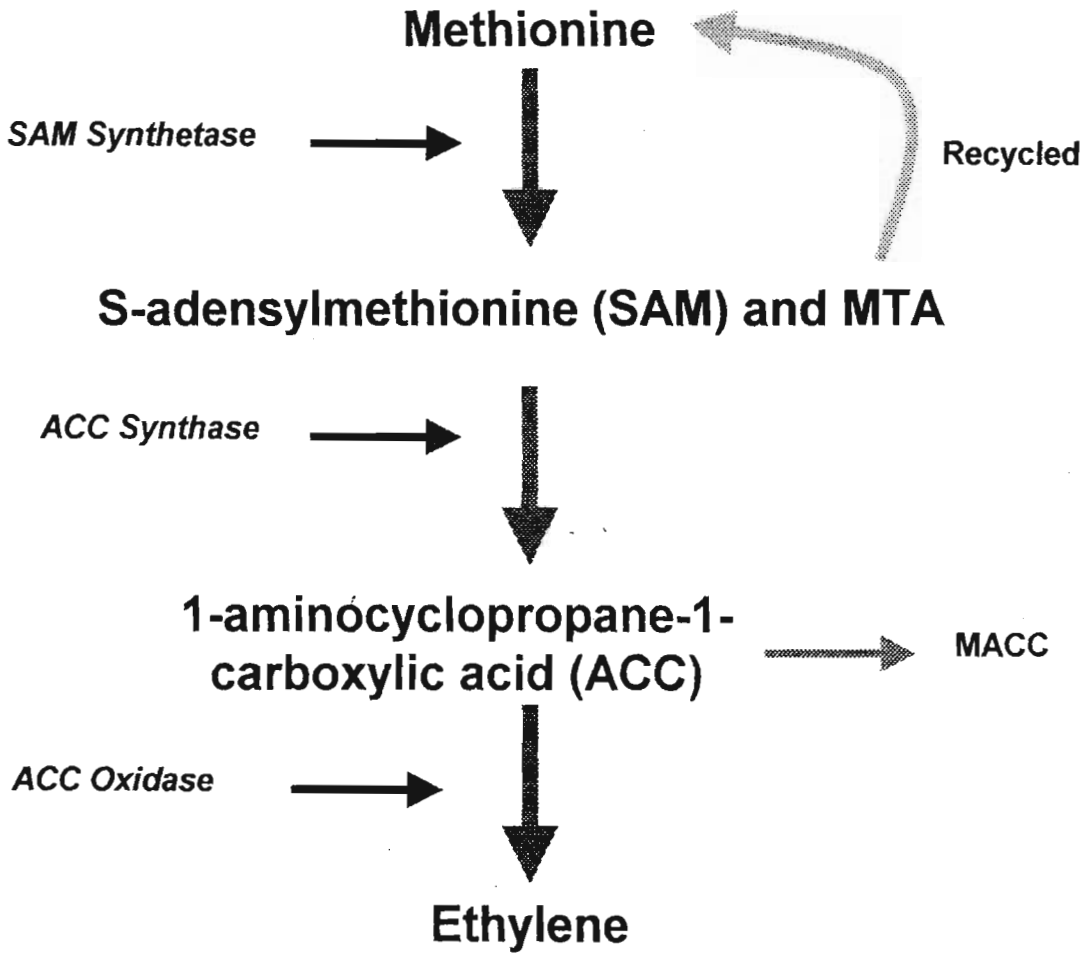


Figure 1.1. The biosynthesis of ethylene. Enzymes are indicated in italics.

in very low levels in young carnation flowers (ABELES, 1972; VAN DER WESTHUIZEN and DE SWART, 1978). As the flower ages, the methionine levels increase. This may be a result of water stress or proteolysis, and is associated with the accumulation of amino acids. Although not directly involved in ethylene production, or flower senescence, it is possible that the methionine increase could cause an increase in ethylene levels (COOK and VAN STADEN, 1988).

Methionine is converted to S-adenosylmethionine (SAM) in the next step of the biosynthetic pathway. (ADAMS and YANG, 1977). The enzyme S-adenosylmethionine synthetase (SAM synthetase) mediates the reaction. The only role SAM was previously known to play in flower senescence was in the production of polyamines, which have protective effects in plant systems (DOWNS and LOVELL, 1986b; NICHOLS and MANNING, 1986; COOK and VAN STADEN, 1988). It has been proposed that if the polyamine pathway is blocked, SAM becomes available, and is channelled into ethylene production (COOK and VAN STADEN, 1988). The by-product of SAM formation is 5'-methylthioadenosine (MTA). This MTA can be recycled to methionine, thus allowing high rates of ethylene production, even if methionine concentrations are low (YANG and HOFFMAN, 1984; ABELES, MORGAN and SALTVEIT, 1992; YANG and DONG, 1993; VAN ALTVORST and BOVY, 1995).

The next compound in the pathway is 1-aminocyclopropane-1-carboxylic acid (ACC), and the conversion of SAM to ACC is mediated by the enzyme 1-aminocyclopronane-1-carboxyle synthase (ACC synthase) (YU, ADAMS and YANG, 1979; YANG, 1980; ADAMS and YANG, 1981; YANG and HOFFMAN, 1984; YIP, DONG and YANG, 1991). ACC synthase is a pyridoxal enzyme (YU, ADAMS and

YANG, 1979), and probably occurs in the cytoplasm, as it is soluble (BOLLER, HERNER and KENDE, 1979; YIP, DONG and YANG, 1991; KENDE, 1993). The enzyme facilitates the elimination of a proton from the α-carbon, resulting in a carbonium ion. A positive sulphonium ion destabilises the molecule thermodynamically, thereby causing an intermolecular nucleophile displacement reaction by the carbonium ion, yielding ACC to MTA (YANG and HOFFMAN, 1984; COOK, 1985; MATTOO and AHARONI, 1988). SAM thereby provides a positive sulphonium ion to facilitate the γ elimination reaction in the formation of ACC (COOK, 1985; MATTOO and AHARONI, 1988).

If exogenous ethylene is applied to petals, there is a huge increase in ACC production in the lower portion of these petals, although not in the upper portion (MOR, HALEVY, SPIEGELSTEIN and MAYAK, 1985). When SAM is applied directly to flowers, there is no premature wilting, which does occur when ACC is added to the flowers. Therefore, the conversion of SAM to ACC is thought to be a rate-limiting factor in the biosynthesis of ethylene (MOR and REID, 1980; MOR, HALEVY, SPIEGELSTEIN and MAYAK, 1985; COOK and VAN STADEN, 1988).

ACC is the direct precursor of ethylene (ADAMS and YANG, 1979; WIESENDANGER, MARTINONI, BOLLER and ARIGONI, 1986). At harvesting, the ACC content of flowers is low, and increases as the flowers senesce (HSEIH and SACALIS, 1986). The greatest increase in ACC content of the tissues was recorded at the same time as the greatest increase in ethylene production (BUFLER, MOR, REID and YANG, 1980). However, ACC levels still increase in the tissue after ethylene levels begin to decrease. It is possible that this is a result of a more rapid decrease in the conversion rate of ACC to ethylene, than the action of ACC synthase (BUFLER, MOR,

REID and YANG, 1980). The conversion of ACC to ethylene is therefore also a rate-limiting step in ethylene synthesis (COOK and VAN STADEN, 1988). ACC content increases are not uniform throughout the tissues of the flower. ACC levels in the petals do not increase as rapidly as they do in other organs, suggesting that the other floral parts may act as an ACC source for the petals (BUFLER, MOR, REID and YANG, 1980; HSEIH and SACALIS, 1986; COOK and VAN STADEN, 1988).

Not all the ACC present in the system is destined for conversion to ethylene. It can also be converted to a storage product N-malonyl-1-aminocyclopropane-1-carboxylic acid (MACC). This MACC can rapidly revert to ACC (PEISER, 1986). MACC is formed by combining ACC with a co-factor malonyl-Co-A. The combination is mediated by the enzyme ACC malonyl transferase (PEISER, 1986). It is possible the MACC, and any other ACC-conjugates that accumulate in the tissues, may act as a source for the increase in ACC content in mature tissues (WHITEHEAD, HALEVY and REID, 1984).

The final step in the pathway is the conversion of ACC to ethylene, which is an enzyme-mediated reaction. As ACC synthase has been shown to be a rate limiting step for ethylene biosynthesis, this step was originally thought of as the "fine control" for ethylene formation (YANG and HOFFMAN, 1984). However, the activity of the enzyme is in fact enhanced by increased levels of ethylene (LUI, HOFFMAN and YANG, 1985), and the conversion of ACC to ethylene is now also considered a rate limiting step in ethylene production (COOK and VAN STADEN, 1988). Until recently, the exact identity of the enzyme was not known. It was identified only as the ethylene forming enzyme (EFE). EFE was thought to be a highly structured compound, dependant on oxygen (LIEBERMAN, 1979; McKOEN and YANG, 1983). Identifying EFE was a problem

because any system that contains or produces oxidants has the capacity to convert ACC to ethylene (KENDE, 1989). In order to determine between natural and artificial ACC-dependent ethylene-forming activities, it is necessary to consider the structure of ACC.

ACC contains a ring structure consisting of four hydrogen atoms, which can be replaced, one at a time, with an ethyl group to yield four stereoisomers of an ACC analogue. Artificial ethylene-forming systems do not show this stereospecificity (HOFFMAN, YANG, ICHIHARA and SAKAMURA, 1982; KENDE, 1989). The exact identity of EFE was eventually determined using molecular cloning. The enzyme could not be isolated using conventional methods, but was identified by functional expression of a ripening-related gene (KENDE, 1993). The process was based on a systematic search for, and characterisation of, mRNAs that were expressed under conditions of enhanced ethylene production, such as during fruit ripening and as after wounding (KENDE, 1993). Work was done using tomato fruits at various stages of ripening. A clone was constructed, designated pTOM13, which appeared to be involved in increased ethylene synthesis (GRIERSON, SLATER, SPEIRS and TUCKER, 1985; SLATER, MAUNDERS, EDWARDS, SCHUCH and GRIERSON, 1985; SMITH, SLATER and GRIERSON, 1986). A great deal of work followed, involving the construction of clones from many different plant types, including carnations. Eventually genes were isolated, and it was possible to produce EFE activity with the correct stereospecificty (MAUNDERS, HOLDSWORTH, SLATER, KNAPP and BIRD, 1987; HOLDSWORTH, BIRD, RAY, SCHUCN and GRIERSON, 1987; HOLDSWORTH, SCHUCH and GRIERSON, 1987; HOLDSWORTH, SCHUCH and GRIERSON, 1988; HAMILTON, LYCETT and GRIERSON, 1990; HAMILTON, BOUZAYEN and

GRIERSON, 1991; FELIX, GROSSKOPF, REGENASS, BASSE and BOILER, 1991; WANG and WOODSON, 1991). The ethylene-forming enzyme was finally identified as ACC oxidase (VERVERIDIS and JOHN, 1991; SMITH, VERVERIDIS and JOHN, 1992). The conversion of ACC to ethylene by EFE was proposed to involve an amine radical cation via two sequential one-electron oxidation steps, with the final products being ethylene, cyanide and carbon dioxide (PIRRUNG, 1983; PEISER, WANG, HOFFMAN, YANG, LUI and WALSH, 1984; MATTOO and AHARONI, 1988; YIP and YANG, 1988). More recently, the action of ACC oxidase has been proposed to involve the conversion of ACC, oxygen and ascorbate to ethylene, cyanide, carbon dioxide, dehydroascorbate and water in the presence of carbon dioxide and iron (DONG, FERN'ANDEZ-MACULET and YANG, 1992). The exact mechanism of ACC oxidase action is still not fully understood.

ACC oxidase is associated with the inside face of the tonoplast (BOROCHOV and ADAM, 1984). Activity of this enzyme depended on membrane integrity, because of the requirement of a transmembrane ion gradient (MAYNE and KENDE, 1986; KENDE, 1989). However, in addition to membrane integrity, ACC oxidase also requires tissue integrity (PORTER, BORLAKOGLU and JOHN, 1986). It seems that some cells have an internal and external site for ACC oxidase activity, while others only have an internal site (KENDE, 1993). The activity of ACC oxidase increases in carnation flower tissue during senescence, and is much higher than in young or mature tissues (MAYAK, LEGGE and THOMPSON, 1983; MANNING, 1986a; MANNING, 1986b). In petals, ACC oxidase is only found in the lower portions of the petal. The top, "flag" portion of the petals is unable to manufacture ethylene, but is still responsive to it (MAYAK and ADAMS, 1984).

1.3.1.2. Pollination-induced ethylene

There is a great deal of interaction between the various organs of senescing carnation flowers, and it appears that this was developed to assist in pollination (COOK and VAN STADEN, 1988). The role of flowers is to encourage pollination. So, not surprisingly, after pollination has occurred, the longevity of flowers is greatly reduced. This is advantageous to the flowers. The growth of excessive pollen tubes may result in competition for stylar food reserves, which are used exclusively to fuel the growing pollen tubes (WHITEHEAD, FUJINO and REID, 1983b). The maintenance of the elaborate floral structures is extremely costly in terms of respiratory energy used, as well as the transportation of water. Minimisation of this energy use is to the plant's advantage (STEAD, 1992; VAN ALTVORST and BOVY, 1995).

Pollination results in accelerated ethylene production (FIGURE 1.2), early irreversible wilting of the petals within one to two days and ovary growth (NICHOLS, 1977a; NICHOLS, BUFLER, MOR, FUJINO and REID, 1983; COOK and VAN STADEN, 1988; VAN ALTVORST and BOVY, 1995). Ethylene production originates from the stigma, style, receptacle and petals, particularly at the base (NICHOLS, 1977a; VEEN, 1979; NICHOLS, BUFLER, MOR, FUJINO and REID, 1983). The original source of ACC appears to be the exine of foreign pollen grains. This ACC results in the stigma producing ethylene within half an hour of the pollen landing (WHITEHEAD, FUJINO, and REID, 1983a; MANNING, 1986b; COOK and VAN STADEN, 1988). Within three hours of pollination, the production of ethylene by the stigma has increased ten fold (NICHOLS, BUFLER, MOR, FUJINO and REID, 1983). Considering how huge this ethylene increase is, ACC levels are extremely low within the stigma compared to other floral parts (WHITEHEAD, FUJINO and REID, 1983a). A possible reason for the low

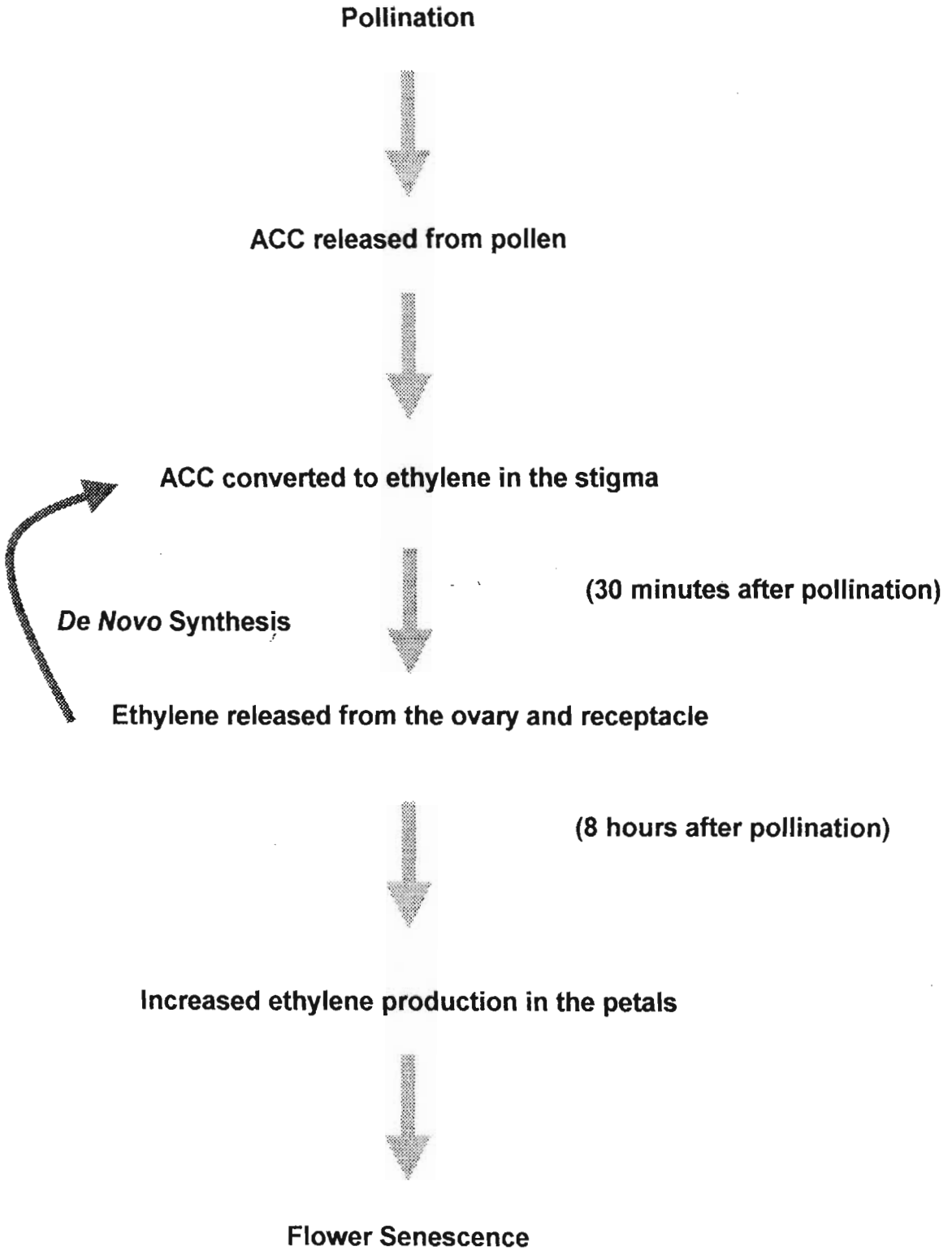


Figure 1.2. A schematic representation of pollination-induced ethylene production.

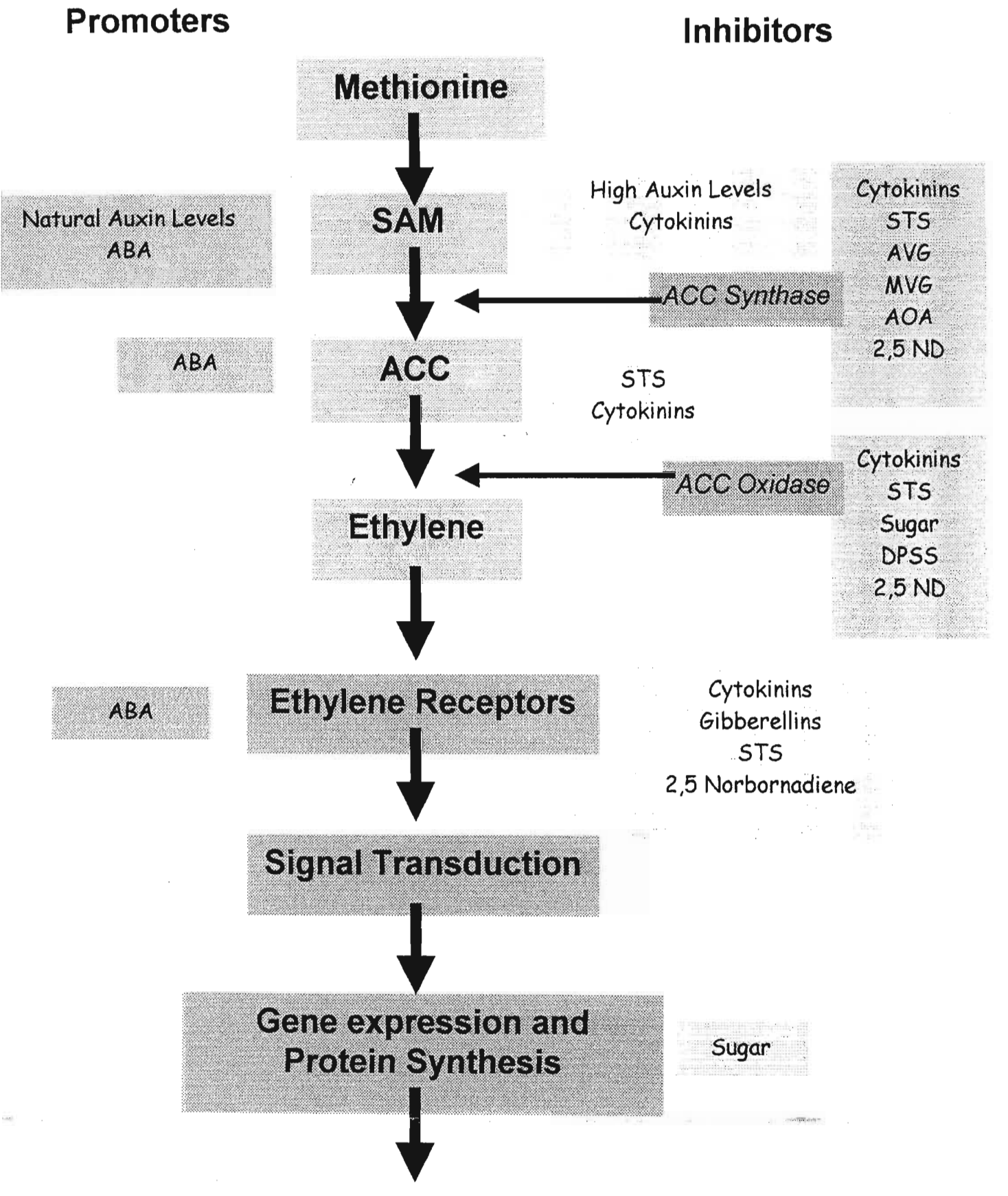
levels of ACC in the stigma is that levels of ACC oxidase are elevated. This will result in any ACC present within the stigma being rapidly converted to ethylene (MANNING, 1986b). The ACC from the pollen appears to only be involved in the initial burst in ethylene production by the stigma, which occurs before the pollen has even germinated. Thereafter, factors such as the *de novo* synthesis of enzymes, transportation of ACC, and stimulated production of ACC oxidase could be activated to continue ethylene production (WHITEHEAD, FUJINO and REID, 1983a; WHITEHEAD, FUJINO and REID, 1983b; COOK and VAN STADEN, 1988; REID and WU, 1992). Apart from the stigma, around the time of pollination the ovary and receptacle also show a small increase in ethylene production. Ethylene production in the petals also increases, but only eight hours after pollination (NICHOLS, BUFLER, MOR, FUJINO and REID, 1983). Rapid increases in ethylene production after pollination suggests the movement of a stimulus from the stigma to other floral organs, such as a transmissible factor for ethylene production (VAN ALTVORST and BOVY, 1995). Both ethylene and ACC could be responsible for this stimulus. There is evidence of an, as yet unidentified, sensitivity factor that moves from the pollinated ovary to the petals. Research indicates that short chain saturated fatty acids are involved (HALEVY, 1986; REID and WU, 1992).

It is important to note that standard carnations are imperfect, in that they have been selectively bred to have no functional stamens (SACALIS and LEE, 1985). The advantage of this is that flowers sold for commercial purposes will not experience accelerated senescence due to pollination. Therefore, pollination-induced ethylene production is no longer a major factor in longevity studies of cut carnation flowers. The relationship between pollination and ethylene production does, however, indicate a highly successful system in terms of energy utilisation (COOK and VAN STADEN,

1988).

1.3.1.3. Mode of ethylene action

The mode of ethylene action in the carnation flower has not been completely elucidated (COOK and VAN STADEN, 1988). It has been proposed that ethylene molecules bind to a specific binding site. This interaction results in a secondary messenger being released. The messenger is a molecule which either itself, or through the production of subsequent messenger molecules, results in the transcription of new m-RNA material from the genome (SISLER, REID and FUJINO, 1983; COOK and VAN STADEN, 1988; WOLTERING, OVERBEEK and HARREN, 1991). The proteins encoded by the RNA are enzymes that result in the symptoms of ethylene action (FIGURE 3.1), such as irreversible petal wilting and ovary growth (SISLER. REID and FUJINO, 1983; SISLER, REID and YANG, 1986; COOK and VAN STADEN, 1988). In order to prove the existence of the binding sites, isotope competition techniques were used. Treatment with silver ions, known to inhibit ethylene action, resulted in ethylene binding being inhibited, and carnation senescence being delayed (VEEN, 1979; SISLER, REID and FUJINO, 1983; SISLER, REID and YANG, 1986; COOK and VAN STADEN, 1988). In other plants, there is extensive evidence that ethylene receptors are associated with membranes. There is only indirect evidence for this in carnation flowers. Electron microscopy indicates that applied silver ions, thought to combine with the binding sites, accumulated in the vicinity of the middle lamella and the plasma membrane of the receptacle (VEEN, HENSTRA and DE BRUYN, 1980; BROWN, LEGGE, SISLER, BAKER and THOMPSON, 1986). The number of ethylene binding sites changes as the flower ages. The peak in ethylene binding precedes the climacteric-like rise in ethylene production, even when the biosynthesis of ethylene is prevented


Promoters
Inhibitors
Methionine
SAM
Natural Auxin Levels
ABA
High Auxin Levels
Cytokinins
Cytokinins
STS
AVG
MVG
AOA
2,5 ND
ACC Synthase
ABA
ACC
STS
Cytokinins
ACC Oxidase
Cytokinins
STS
Sugar
DPSS
2,5 ND
Ethylene
ABA
Ethylene Receptors
Cytokinins
Gibberellins
STS
2,5 Norbornadiene
Signal Transduction
Gene expression and
Protein Synthesis
Sugar

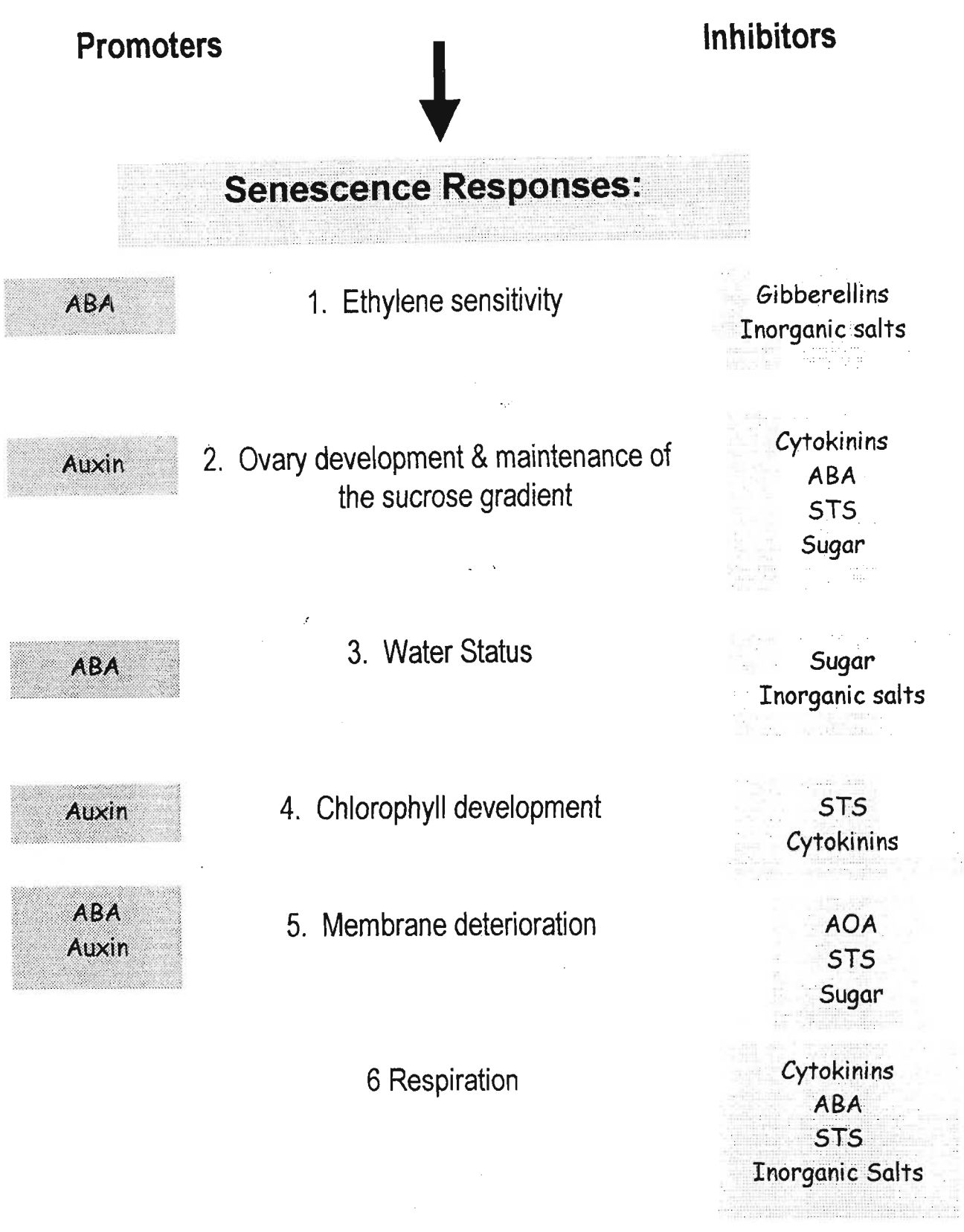


Figure 1.3. The role of various promoters and inhibitors of senescence on both ethylene formation and ethylene action. Enzymes are indicated in italics.

using an inhibitor (BROWN, LEGGE, SISLER, BAKER and THOMPSON, 1986). Thereafter the number of ethylene binding sites declines with the age of the flower. This is related to extensive membrane breakdown as the flowers senescence, and is indicated by a marked decrease in phospholipid phosphate in the petals (THOMPSON, MAYAK, SHINITZKY and HALEVY, 1982; BROWN, LEGGE, SISLER, BAKER and THOMPSON, 1986).

1.3.1.4. Ethylene sensitivity and auto-catalytic ethylene production

The sensitivity of carnation flowers to ethylene is a regulating factor in carnation flower senescence. Exposure of the plant to ethylene results in an autocatalytic induction of several enzymes in the synthesis of ethylene. This can be accounted for by changes in sensitivity of tissues to ethylene. Sensitivity to ethylene increases with age, as older petals will senesce in response to a lower concentration of ethylene than younger petals (VAN ALTVORST and BOVY, 1995). This is not related to an increase in ethylene binding capacity (BROWN, LEGGE, SISLER, BAKER and THOMPSON, 1986), and it has been suggested that the increased ethylene sensitivity may result from the production of a sensitivity factor (WHITEHEAD and VASILJEVIC, 1993; VAN ALTVORST and BOVY, 1995). It is possible that short chain fatty acids may be involved, as pollination induces both their synthesis, and an increase in sensitivity to ethylene (HALEVY, 1986; VAN ALTVORST and BOVY, 1995). Octanoic acid is an example of a short chain fatty acid. If octanoic acid is applied to the style of unpollinated flowers, there is an increase in sensitivity to ethylene, as well as marked petal senescence. The increase in sensitivity to ethylene in preclimacteric flowers is associated with an accumulation of short-chain fatty acids (containing seven to ten carbons) in the petals during the early stages of senescence.

These acids may increase ethylene-binding affinity by altering membrane properties. When ethylene action was suppressed by silver ions, so were the levels of short chain fatty acids (WOODSON and VASILJEVIC, 1993; VAN ALTVORST and BOVY, 1995).

1.3.2. Abscisic acid

Like ethylene, abscisic acid (ABA) accelerates carnation flower senescence (FIGURE 1.3). Endogenous ABA levels increase in senescing flowers (MAYAK and DILLEY, 1976a; MAYAK and DILLEY, 1976b; NOWAK and VEEN, 1982; EZE, MAYAK, THOMPSON and DUMBROFF, 1986; NICHOLS and MANNING, 1986). This increase coincides with an increase in ethylene production, as well as the onset of irreversible petal wilting. Endogenous levels of ABA in the petals and calyx increase from the first day of harvest (NOWAK and VEEN, 1982). In the ovary, ABA content increases immediately after harvest, reaches a peak after three to four days, and then declines. It is possible that this peak level of ABA is associated with increased sink activity of the ovary, as ABA has been shown to be involved in the regulation of assimilate transport (TIETZ, LUDEWIG, DINGKUHN and DÖRFFLING, 1981). The application of ABA to cut carnation flowers advances the autocatalytic production of ethylene (FIGURE 1.3), unless the production or action of ethylene is inhibited (HALEVY, MAYAK, TIROSCH, SPIEGELSTEIN and KOFRANEK, 1974; MAYAK and DILLEY, 1976b; RONEN and MAYAK, 1981; NOWAK and VEEN, 1982). Ethylene biosynthesis, from the action of ACC synthase onwards, is necessary in order for ABA to cause petal wilting. There is confusion as to how ABA accumulation in the tissues coincides with the ethylene climacteric. It has been reported that the climacteric ethylene rise only occurs after the accumulation of ABA in the ovary. It is therefore possible that ABA may induce ethylene production (NOWAK and VEEN, 1982). However, EZE and co-workers (1986) found

the rise in ABA occurred parallel to the rise in ethylene, and were therefore unable to clearly define whether the initial ABA increase followed the ethylene emanation or not (EZE, MAYAK, THOMPSON and DUMBROFF, 1986).

When ABA was applied to carnation flowers, in conjunction with silver ions, ethylene biosynthesis occurred, but there was no ethylene action (RONEN and MAYAK, 1981; NOWAK and VEEN, 1982; COOK and VAN STADEN, 1988). This suggests that ethylene action may be necessary to stimulate ABA synthesis. Membrane deterioration, and a change in the water status of the harvested carnation, was apparent well before the natural rise of ethylene and ABA. It therefore appears that increases in ethylene production and endogenous ABA do not initiate senescence (EZE, MAYAK, THOMPSON and DUMBROFF, 1986). As silver treatment delays senescence processes, this may explain the absence of any increase in these two hormones. The ABA increase may be a response to changes in water status (BOROCHOV, TIROSH and HALEVY, 1976; BOROCHOV, MAYAK and BROWN, 1982).

ABA has been implicated in increased sensitivity of senescing cut carnation flowers to ethylene (MAYAK and DILLEY, 1976a; RONEN and MAYAK, 1981; NOWAK and VEEN, 1982). There are several reasons for this. The application of ethylene and ABA in combination resulted in rapid senescence, greater than the application of either compound individually could account for. When ethylene action was inhibited, ABA did not promote flower senescence (RONEN and MAYAK, 1981). Ethylene sensitivity is known to increase under conditions of water stress (MAYAK and DILLEY, 1976a), and ABA levels also increase during water stress. ABA may be one of the hormones that change the ethylene sensitivity of the flower in response to environmental conditions

(BOROCHOV, TIROSCH and HALEVY, 1976; BOROCHOV, MAYAK and BROWN, 1982).

1.3.3. Auxin

Auxin is a plant hormone that is not commonly associated with flower senescence, as it functions mainly in cell wall elongation and cell division. However, as the phyto-hormones always act correlatively, the effect of auxin on flower longevity cannot be ignored. Moderate concentrations of the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) promoted ethylene production, and so induced senescence (YU and YANG, 1979) (FIGURE 1.3). A much higher concentration (500 mg.ℓ^{-1}) retarded petal senescence and inhibited ethylene production (SACALIS and NICHOLS, 1980). However, the levels that retarded senescence were much greater than the normally occurring levels of the hormone. The levels that promoted senescence were close to the endogenous levels of auxin in the system. The natural effect of auxin in cut carnation flowers is assumed to reduce their longevity.

Auxin accelerates senescence by stimulating ACC synthase activity in other plant systems (YU and YANG, 1979), and promoting both ACC and ethylene levels in the petals, ovary and receptacle in cut carnation flowers (NICHOLS and MANNING, 1986). The interaction between ethylene biosynthesis and auxin has been particularly investigated in the petals of senescing carnation flowers. It has been suggested that the auxin indole acetic acid (IAA) is involved in the transportation of ACC to the petals (SACALIS, WULSTER and JANES, 1983). Exogenously applied IAA is found in all parts of the petal, but there is only increased senescence at the petal bases, where ACC is synthesised. Some form of messenger, probably ethylene, would then have to migrate

to the distal portion of the petal, in order to cause irreversible petal wilting. It is therefore clear that if endogenous levels of IAA increased in the petal, ACC synthesis, and thus ethylene biosynthesis, would also increase, and irreversible petal wilting would take place (AHARONI, ANDERSON and LIEBERMAN, 1979; COOK and VAN STADEN, 1988).

Aside from the promotion of ethylene biosynthesis, auxin is also involved in assimilate mobilisation. When an isolated floral head was treated with IAA, movement of the ^{14}C-labelled assimilates continued (JEFFCOAT and HARRIS, 1972). Endogenous levels of auxin may regulate the shift in the carbohydrate sink from the petals to the ovary (COOK and VAN STADEN, 1988). In *in vitro* studies of isolated ovaries placed onto a media containing the auxin naphthalene acetic acid (NAA), the ovary greened and increased in fresh mass, as is seen in senescing flowers. There was also an increase in the efficiency of sucrose uptake, due to greater invertase activity that would serve to maintain the sucrose gradient. Another result of this treatment is the stimulation of chloroplast development in the ovary walls, indicating that the photosynthetic capacity of the senescing ovary may also contribute to the carbohydrate accumulation (COOK and VAN STADEN, 1986).

1.3.4. Cytokinins

That cytokinins delay cut carnation flower senescence is well documented (MACLEAN and DEDOLPH, 1962; HEIDE and ØYDVIN, 1969; MAYAK and KOFRANEK, 1976; EISINGER, 1977; JEFFCOAT, 1977; EISINGER, 1982; COOK and VAN STADEN, 1988). The senescence-delaying action of cytokinins is ubiquitous throughout the flowering plant kingdom (COOK and VAN STADEN, 1988). In leaf tissue

cytokinins delay the loss in chlorophyll associated with senescence, as well as stimulating protein synthesis and stabilising respiration (SACHER, 1973; HORGAN, 1984). The action of cytokinins in flowers has not been as clearly elucidated (FIGURE 1.3). Cytokinins, particularly benzyladenine (BA), lower the respiration rate of cut carnation flowers, repressing the normal climacteric respiration increase associated with ethylene production (MACLEAN and DEDOLPH, 1962). It is also possible that increased membrane integrity in the final stages of senescence, as seen in cytokinin-treated flowers, is a result of increased respirable material being present in the system (HEIDE and ØYDVIN, 1969; PAULIN, 1986).

Another physiological system affected by cytokinins is ethylene production. Cytokinins are able to block the biosynthesis of ethylene (EISINGER, 1977; MOR, SPIEGELSTEIN and HALEVY, 1983; RASCHE and EISINGER, 1984; COOK, RASCHE and EISINGER, 1985; COOK and VAN STADEN, 1988) and its action (EISINGER, 1977; COOK, RASCHE and EISINGER, 1985). The point at which cytokinins effect the ethylene biosynthetic pathway is still unclear. Benzyladenine (BA) effects ACC synthase activity (MOR, SPIEGELSTEIN and HALEVY, 1983). It also causes a reduction in the levels of ACC, the action of ACC oxidase (EISINGER, 1982; MOR, SPIEGELSTEIN and HALEVY, 1983; COOK, RASCHE and EISINGER, 1985) and resultant ethylene production (EISINGER 1977; EISINGER, 1982). This does not, however, rule out the possibility that the affect may occur earlier in the pathway, such as in the conversion of SAM to ACC. This may explain why cytokinin treatment is only effective if applied very early in the post-harvest life of the flower, before the ACC or ethylene increases occur (COOK and VAN STADEN, 1988; VAN STADEN, 1995).

Flowers remain photosynthetic units, even after removal from the parent plant (COOK and VAN STADEN, 1983). However, the maintenance of chlorophyll levels due to cytokinin treatment of these photosynthetic organs has never been considered as reason for cytokinin-induced longevity (COOK and VAN STADEN, 1988). It may be more than coincidence that there are many similarities between the pattern of cytokinin translocation within the flower and that of sucrose (COOK and VAN STADEN, 1983). Cytokinins have been implicated in the translocatory movement of assimilates within the flower (VAN STADEN and DIMALLA, 1980). It is not clear whether cytokinins cause the movement of sucrose to the ovary, or visa versa. The localised cytokinin occurrence in the ovary is thought to cause a preferential carbohydrate sink (VAN STADEN and DIMALLA, 1980). However, an injection of zeatin into the ovary did not result in more than minor sucrose mobilisation to the ovary (COOK, 1985). The inclusion of cytokinins into culture media also failed to result in dry weight increases (COOK and VAN STADEN, 1986; FEATONBY-SMITH, VAN STADEN and COOK, 1987). The application of silver ions to the system inhibited sucrose accumulation in the ovary, as well as preventing cytokinin accumulation (COOK and VAN STADEN, 1983; KELLY, STABY and CHISM, 1985). Sucrose is required for effective kinetin transportation (MAYAK and DILEY, 1976a), so it is possible that the accumulation of cytokinins in the ovary of senescing carnation flowers may be due to sucrose facilitating its mobility by forming cytokinin glucosides (COOK and VAN STADEN, 1988; VAN STADEN, 1995).

It was originally proposed that the role of cytokinins within senescing cut carnation flowers is a natural one. As the flower senesces, the levels of cytokinins decrease, acting as a senescence trigger which leads to increased ethylene production

(EISINGER, 1977). However, it is now generally accepted, although not comprehensively proven, that the roots are the source of cytokinins within the plant (VAN STADEN and DAVEY, 1979). Thus the removal of the flower from the parent plant would limit the source of cytokinins within the system. Yet when cytokinin levels in the system were measured, levels declined until after irreversible petal wilting occurred. Thereafter they increased slightly, resulting in higher cytokinin levels than were found in non-senescing tissues (VAN STADEN and DIMALLA, 1980). The source of this cytokinin increase can only be speculated to be the developing seeds (VAN STADEN, FEATONBY-SMITH, MAYAK, SPIEGELSTEIN and HALEVY, 1987).

The degree of flower senescence retardation depends on the particular cytokinin involved. This may be because each cytokinin has a different affinity for the same receptors (VAN DER KRIEKEN, CROES, SMULDERS and WULLEMS, 1990), or is perhaps due to different metabolic pathways in their formation (VAN STADEN and BOSSE', 1989). Many different endogenous cytokinins have been detected in senescing carnation flowers, and one would expect the cytokinin pool to change during the course of senescence. Each different cytokinin has a physiological action within the system, so as the cytokinin pool changes, so the role played by individual cytokinins in the senescing flower will change. Among the endogenous cytokinins identified at this point are *trans*-zeatin, *trans*-ribosylzeatin, dihydrozeatin, *iso*-pentenyladenine, zeatin-O-glucoside and ribosylzeatin-O-glucoside. The petals contained *iso*-penteyladenosine, but not ribosylzeatin-O-glucoside (FEATONBY-SMITH, VAN STADEN and HOFMAN, 1987; VAN STADEN, 1995).

1.3.5. Gibberellins

Original investigations into the action of applied gibberellins in cut carnation flowers showed that there was significant change in longevity (NICHOLS, 1968). In a later study, however, when detached petals were fed gibberellin alone, or in combination with sucrose, longevity was increased (GARROD and HARRIS, 1978; SACALIS, 1986). In attached flowers, gibberellins are thought to act by stimulating assimilate mobilisation from the stems and leaves to the flower head (FIGURE 1.3), as levels of gibberellin in the flower are higher than in any other tissue (JEFFCOAT and HARRIS, 1972). When flowers were detached, applied gibberellin partially compensated for the loss in assimilate accumulation (NICHOLS and MANNING, 1986), and prevented the loss in fresh weight normally associated with senescence (SAKS and VAN STADEN, 1993a, 1993b). The majority of responses within the ovary to this hormone are therefore primarily associated with growth, rather than increasing its longevity. Gibberellin application was also shown to reduce the sensitivity of the flowers to exogenously applied ethylene. This prevents normal irreversible petal wilting (SAKS and VAN STADEN, 1993a). When gibberellin was applied directly to the style, the prevention of ethylene formation by gibberellin application was reported (SAKS and VAN STADEN, 1993b). During senescence there is normally an increase in membrane permeability in the later stages of senescence, and this is delayed in gibberellin-treated flowers (SAKS and VAN STADEN, 1993a). It was suggested that gibberellins may play more of a regulating role in flower senescence than a specific retardation role (COOK and VAN STADEN, 1988). In truth the real role of these compounds in flower senescence still needs to be thoroughly elucidated (VAN STADEN, 1995).

1.4. Factors affecting cut carnation flower longevity

A number of factors may influence the post-harvest life of cut carnation flowers. These include pre-harvest conditions, post-harvest treatments and the cultivar of the flowers.

1.4.1. Pre-harvest conditions

Growing conditions of the carnation flowers, such as the length and quality of light exposure, temperature fluctuations and reduced carbohydrate supplies can effect the resultant vase-life of the cut flowers. Any pathogens, such as micro-organism invasion or plant diseases, can also affect the longevity of excised organs (KOHL and NELSON, 1965; UPFOLD, 1992). Other growing factors that may exert an influence include nutrient availability, growing media and irrigation, resulting in changes in the carbohydrate status, transport or nutrient uptake of the cut flowers.

The production and sensitivity of the flowers to ethylene may also be affected (HALEVY and MAYAK, 1979; UPFOLD, 1992). For example if plants are exposed to water stress, they may develop an osmotic adaptation that manifests itself as a reduction in sensitivity to ethylene in the harvested plant (MAYAK and KOFRANEK, 1976). Pollination is another pre-harvest event that can have a dramatic effect on the longevity of cut carnation flowers.

As previously discussed, pollination of cut carnation flowers results in ethylene production and accelerated petal wilting (NICHOLS, 1977a; NICHOLS, BUFLER, MOR, FUJINO, and REID, 1983; GOSZCZYNSKA, RUDNICKI and REID, 1985; STEAD, 1992;

VAN ALTVORST and BOVY, 1995). It is therefore necessary to prevent flower pollination as far as possible, although pollination is seldom a problem for commercial cut flower growers, as the anthers and stamens of flowers are generally greatly reduced and non-functional (UPFOLD, 1992).

1.4.2. Post-harvest treatments

Original investigations into chemically-induced longevity increases were based on old wife's tales, such as placing a copper penny, sugar, whiskey and more recently aspirin and bleach into the vase with the water. The commercial benefits of extending the vase-life of cut flowers are huge. Therefore a large number of compounds have been tested on carnation flowers in attempt to increase their longevity. A wide variety of compounds have anti-senescence effects. These range from enzyme inhibitors to uncouplers of phoshorylation, free radical scavengers and relatively simple preservative solutions (BAKER, WANG and TERLIZZI, 1985; COOK, 1985; UPFOLD, 1992). These can be divided into two categories. Natural anti-senescence treatments include compounds that occur within plant tissues naturally, but which delay their senescence when introduced in higher volumes. Artificial post-harvest treatments are synthetic chemicals which are applied to the flowers and their biochemical properties cause a delay in senescence. The effect of these compounds on ethylene, either its biosynthesis, action or the plant's response to it, are shown in FIGURE 1.3.

1.4.2.1. Natural post-harvest treatments

The majority of early preservative solutions were composed mainly of sugar, in an effort to improve water relations within the cut flower, and prevent starvation of the organ after excision from the parent plant. As sucrose is the main translocatory

carbohydrate, it is more commonly applied to cut flowers than either glucose or fructose. Except during the climacteric respiration increase, these applied sugars are stored in the flower, therefore supplementing the assimilate supply used for respiration and other important metabolic functions. The stored sugars also serve to maintain or increase the osmotic potential of the tissue, thereby preventing excessive water loss and irreversible petal wilt. This osmotic property is eventually lost towards the end of corolla life. At this point, osmotic energy decreases more rapidly than can be accounted for by sugar loss, indicating massive cell disruption. This indicates that, even though the carbohydrate status of the flower is extremely important, it is not the sole determining component controlling flower senescence (NICHOLS, 1977b; COOK, 1985). A number of other processes are effected by exogenously applied sugars as well. These include the inhibition of the onset of protein synthesis and respiration (NICHOLS, 1973), the maintenance of mitochondrial structure, and the protection of membrane integrity (HALEVY, 1976). There is a reduction in losses in dry matter from carnation petals after sugar application, and even enhancement of growth (NICHOLS and HO, 1975a), although sugar was not effective in this regard when applied to isolated petals (PAULIN, 1986). Even ethylene synthesis is affected by sugar treatment, with the activity of ACC oxidase being inhibited most effectively by sorbitol and least effectively by glucose (MAYAK and BOROCHOV, 1984). The application of sugar in combination with other preservative compounds has also resulted in increased colour density and floral diameter (BELENSKAYA, KONDRAT'EVA and SMIRNOVA, 1985).

The endogenous role of cytokinins within flower senescence has already been discussed, but it must be reiterated at this point that applied cytokinins are generally considered to act as inhibitors of carnation flower senescence (COOK and VAN

STADEN, 1988). When all of the naturally occurring and synthetic cytokinins were applied to cut carnation flowers, many resulted in longevity increases, although dihydrozeatin gave the best results, whether applied as a pulse treatment or a holding solution (BOSSE' and VAN STADEN, 1989). Benzyl Adenine applied as a low concentration holding solution, has also increased flower longevity (MACLEAN and DEPOLPH, 1962; HEIDE and □YDVIN, 1969; BOSSE' and VAN STADEN, 1989; VAN STADEN, BAYLEY, UPFOLD and DREWES, 1990).

Another natural compound that increases cut flower longevity is ethanol. Applied as a holding solution it can increase the vase-life of cut carnation flowers as much as 250%. This will be discussed in more detail in Section 1.5. and Chapter 2.

1.4.2.2. Artificial post-harvest treatments

Of all the post-harvest treatments known today, none is more important than silver thiosulphate (STS). Silver ions are extremely effective in delaying the senescence progress of cut carnation flowers as they inhibit ethylene action. This discovery was initially made when silver ions were applied to flowers as a bacteriocide (AARTS, 1957). Trials have shown that silver nitrate ($AgNO_3$) effectively delayed senescence in cut flowers when applied as a dip or spray treatment. However, the positively charged Ag^+ became bound to the negatively charges COO^- in the xylem tissue (HALEVY and KOFRANEK, 1977). To overcome the relative immobility of the silver ion, the negatively charged thiosulphate ion was added (VEEN 1979; 1983). The new compound proved to be highly mobile and effective, and was introduced to the commercial cut flower industry in the early 1980's, where it is still used today (STABY, CUNNINGHAM, EISENBERG, BRIDGEN and KELLY, 1978; SPEAR and GLADON, 1982; VEEN, 1983;

COOK, 1985; COOK and VAN STADEN, 1987; CHUNG, LEE and KIM, 1986; UPFOLD, 1992). Applied as a 15 minute pulse-treatment it increases longevity (as much as 300%), prevents ethylene action and ovary enlargement and eliminates the ovary as a sink (DIMALLA and VAN STADEN, 1980). In silver-treated flowers the conversion of ACC to ethylene is inhibited, and a decrease in endogenous ACC levels also occurs. This is due to inhibition of ACC synthase and ACC oxidase activity. There is also a marked decrease in membrane and chlorophyll breakdown (VEEN, 1979; VEEN and KWAKKENBOS, 1982). As for the prevention of ethylene action, it is thought that the silver ions compete for the ethylene binding sites (COOK and VAN STADEN, 1987). However, no individual site, or combination of sites has yet been identified that fits all of the requirements for known anti-senescence effects of STS (COOK and VAN STADEN, 1988).

Two rhizobitoxine analogues, amino-ethoxy vinylglycine (AVG) and methoxy vinylglycine (MVG), are specific inhibitors of ethylene synthesis in cut carnation flowers. These compounds act by blocking the action of the enzyme ACC synthase, effectively delaying senescence (BAKER, WANG, LIEBERMAN and HARDENBURG, 1977; BAKER, LIEBERMAN and ANDERSON, 1978, BAKER, 1983). When pulsed into flowers in increasing concentrations, AVG continually increased the vase-life. While the concentration of AVG required may be higher than that of STS, there are no phytotoxic effects, unlike those seen after the application of high concentrations of STS (SPEAR and GLADON, 1982). Another rhizobitoxine analogue, L-2-amino-4-(2-aminoethoxy) trans-3-butenoic acid also inhibited the production of ethylene and extended carnation flower vase-life, although not as effectively as AVG or MVG. Amino-oxyacetic acid (AOA) inhibits the action of ACC synthase, suppressing both the respiration and

ethylene increases associated with climacteric flowers such as carnations (YANG, 1980; FUJINO, REID and YANG, 1981). It also reduced the senescence-related breakdown of phospholipids and fatty acids in the membranes (PAULIN, BUREAU and DROILLARD, 1985). While cheaper and easier to use than both STS and AVG, it is debatable whether AOA protects the flower against the action of exogenous ethylene (YANG, 1980; PAULIN, BUREAU and DROILLARD, 1985), limiting its use as a commercial post-harvest treatment.

When applied as a gas, 2,5-Norbornadiene (2,5-ND) extends the vase-life of cut carnation flowers significantly. The result was achieved as 2,5-ND completely blocked ethylene action, by competing for ethylene binding sites (SISLER, REID and FUJINO, 1983; SISLER, REID and YANG, 1986; WANG and WOODSON, 1989). The activity of both ACC synthase and ACC oxidase was also reduced (PEISER, 1989). However, 2,5-ND is an extremely unpleasant toxic vapour, and highly insoluble in water, limiting its potential as a post-harvest treatment, regardless of its effectiveness.

Polyamines are effective anti-senescence agents in some plants. This effect is possibly related to their close link with ethylene biosynthesis, as both these compounds have methionine as a precursor. Polyamines have no effect if applied at the commercially useful stage of carnation development. However, they can be applied successfully to unopened buds (UPFOLD and VAN STADEN, 1992).

Certain inorganic salts, such as potassium nitrite (KNO_3) potassium chloride (KCl), potassium sulphate (K_2SO_4), calcium nitrate ($Ca(NO_3)_2$) and ammonium nitrate (NH_4NO_3) extended the longevity of cut carnation flowers, the effect not being limited to

a specific ion. In particular KNO_3 appeared to cause an osmotic adjustment within the flower, lowering the water potential in the petal tissues, and improving its ability to absorb water, a similar effect to sucrose (HALEVY, 1976; HALEVY and MAYAK, 1981). Flowers treated with inorganic salts show a delay in the ethylene and respiration increases, and reduced sensitivity to exogenously applied ethylene.

Applications of CO_2 delayed senescence by acting as an endogenous inhibitor of ethylene (SMITH and PARKER, 1966; MAYAK and DILLEY, 1976b). At concentrations of two to three percent CO_2 prevents damage to the cells caused by exogenously applied ethylene. The overall effect therefore delays, rather than arrests senescence. Similarly ethylene oxide overcomes the effect of exogenously applied ethylene, and suppresses the climacteric ethylene production (NICHOLS, 1977b).

Among the lesser used post-harvest treatments are sodium benzoate, which inhibits ethylene formation (BAKER, WANG, LIEBERMAN and HARDENBURG, 1977), 1,1-dimethyl-4-(phenylsullfonyl)semicarbazide (DPSS), which appears to inhibit ACC oxidase (MIDOH, SAIJOU, MATSUMOTO and IWATA, 1996), 2-aminoisobutric acid (SERRANO, ROMOJARO, CASAS, RIO and ACOSTA, 1990), cis-propenylphosphoric acid (YAMAMOTO, SAITOH, YOKOO, FURUKAWA and OSHIMA, 1992), and 3-amino-1,2,4-triazole (ALTMAN and SOLOMOS, 1993).

1.4.3. The effect of cultivar on carnation cut flower longevity.

The senescence of the cut carnation flower is frequently studied using the cultivar "White Sim". This is because "White Sim" is an internationally available cultivar (COOK,

1985). In "White Sim" flowers the end of the vase-life is indicated by petal in-rolling, accompanied by an increase in the respiration rate and ethylene production (NICHOLS, 1968; MAYAK and DILLEY, 1976b; WU, VAN DOORN and REID, 1991). However, not all cultivars of carnation respond in the same manner as "White Sim" (WU, VAN DOORN and REID, 1991; WU, ZACHARIAS and REID, 1991; SERRANO, ROMOJARO, CASAS and ACOSTA, 1991; MAYAK and TRIOSH, 1993). The flowers of the cultivar "Sandra" senesce without the typical in-rolling of the petals. They show either the normal increase in ethylene production, or a respiratory climacteric during their senescence (WU, VAN DOORN and REID, 1991). The carnation cultivar "Chinera" also has a longer than normal vase-life (FUKUTOME, 1986; WU, VAN DOORN and REID, 1991; WU, ZACHARIAS and REID, 1991). When the senescence of a range of cultivars was studied ("Yellow Candy", "Sandrosa", "Francisco", "White Candy" and "Iury"), it was found that no cultivar responded in exactly the same manner (PUN, ROWE, ROWARTH, BARNES, DAWSON and HEYES, 1999). This suggests that the production of, sensitivity to, or response to ethylene may vary significantly amongst carnation cultivars (WU, VAN DOORN and REID, 1991; WU, ZACHARIAS and REID, 1991; SERRANO, ROMOJARO, CASAS and ACOSTA, 1991; MAYAK and TRIOSH, 1993; PUN, ROWE, ROWARTH, BARNES, DAWSON and HEYES, 1999). It is also possible that genotypic differences can influence the capacity of a flower to synthesise and metabolise ethylene (BRANDT and WOODSON, 1992). However, only the cultivar "Sandra" shows an inability to produce ethylene. Scientific studies are therefore still most often performed using the cultivar "White Sim", as the senescence of this cultivar seems to represent the majority of carnation cultivars.

1.5. Ethanol, acetaldehyde and senescence

1.5.1. Ethanol

Ethyl alcohol, more commonly referred to as ethanol, is the most commonly occurring alcohol in nature. It has the chemical structure CH_3CH_2OH (FIGURE 1.4).

Figure 1.4. The chemical structure of ethanol.

After methyl alcohol (methanol, CH_3OH) it is the simplest member of the organic family of alcohols. The characteristic functional group in this family is the hydroxyl group (OH), attached to the tetrahedral carbon atom. Structurally, one can consider alcohols in two ways, either as the hydroxy derivatives of alkanes, or as the alkyl derivatives of water. The later method of viewing the compounds is generally considered to be correct, as the C-O-H bond angle is similar in size to that of water (SOLOMONS, 1988).

Alcohols are classified into three groups; primary, secondary and tertiary alcohols. The classification is based on the position of the carbon atom to which the hydroxy group is attached. If the carbon is only attached to one other carbon, it is said to be a primary carbon, and the alcohol, a primary alcohol. If however, the carbon atom to

which the hydroxyl group is attached has two other carbon atoms attached, the compound is a secondary alcohol, and so on. Ethanol is an example of a primary alcohol (SOLOMONS, 1988). Ethanol is liquid at room temperature, having a melting point of –117°C and a boiling point of 78.3°C. It has a low density, 0.789 $g.m\ell^{-1}$, is soluble in water and is highly flammable (BONNER and VARNER, 1965).

Ethanol is biosynthesised during plant metabolism as part of the Embden-Meyerhof-Parnas (EMP) glycolytic conversion of hexose to pyruvate in the absence of oxygen. Pyruvate is converted to lactate under aerobic conditions, or to acetaldehyde and ethanol under anaerobic conditions. The enzymes required for the process are pyruvate carboxylase and alcohol dehydrogenase (FIGURE 1.5). The production of ethanol from acetaldehyde is a reversible reaction, mediated by the same enzyme, if CO_2 is reintroduced to the system (BONNER and VARNER, 1965). Ethanol is also produced in biological systems by the fermentation of sugars from plant material, in the presence of yeast. Ethanol is the alcohol found in wine, beer and other alcoholic beverages, where it is produced by the fermentation process. The synthesis of ethanol, in the form of wine, from the fermentation of sugar and fruit juice was probably man's first accomplishment in organic chemistry, as long ago as 4000 BC. Adding yeast to a mixture of sugars and water carries out fermentation. Yeast contains enzymes that promote a long series of reactions that ultimately convert a simple sugar to ethanol and CO_2 (SOLOMONS, 1988).

In order to obtain ethanol for laboratory work, fermentation is not a suitable process however. In fermentation of sugars, using yeast, the solution that is recovered is only 12 - 15% ethanol, as concentrations greater than this will result in the death of

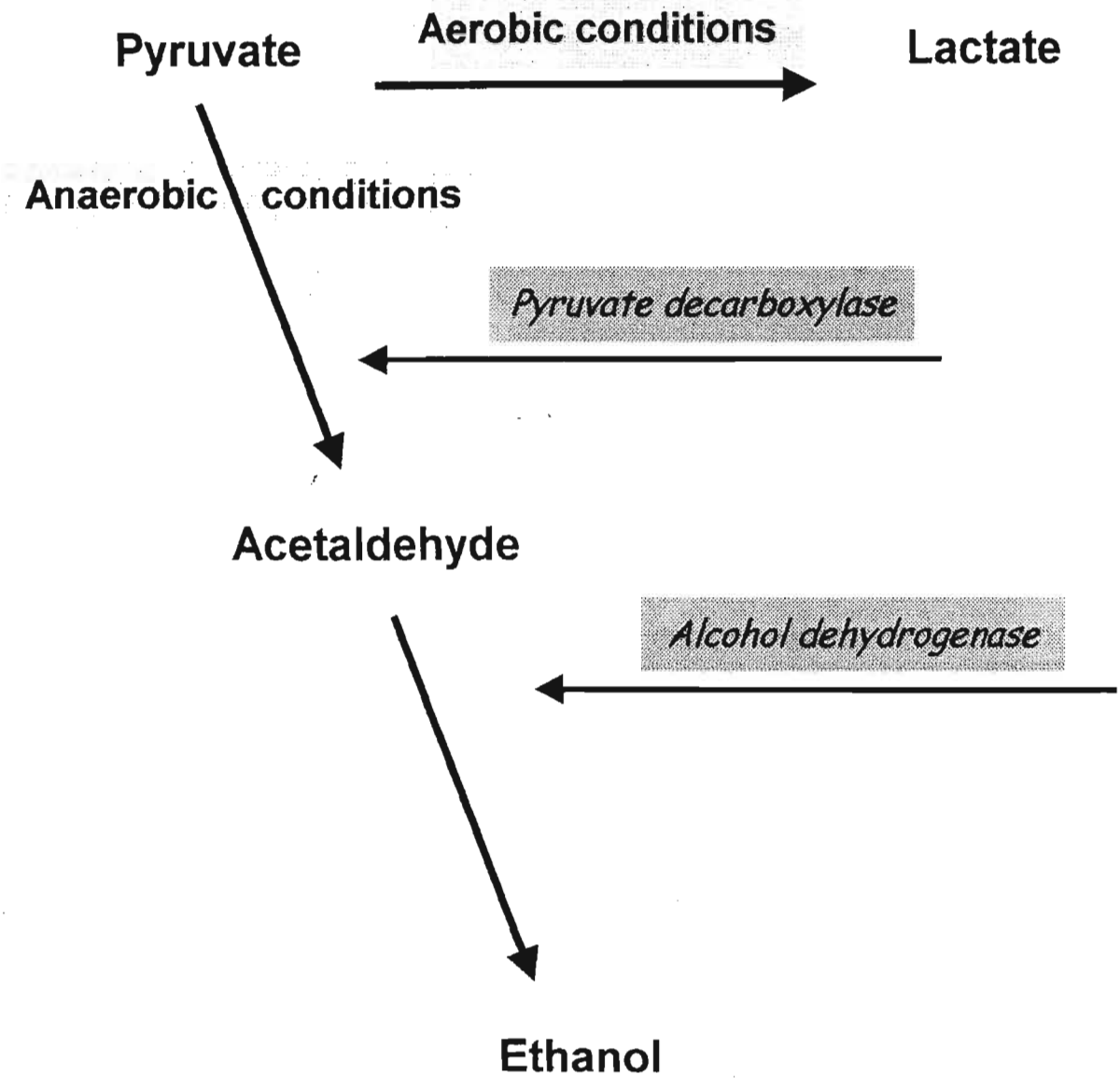


Figure 1.5. A schematic representation of the formation of ethanol and lactate from pyruvate.

the yeast. In order to obtain a strong solution the liquid must be distilled. The distillation of a naturally-produced solution will not yield greater than 95% ethanol, which is referred to as pure alcohol. In order to obtain 100% ethanol, it is necessary to add benzene, and redistill the solution. The benzene will form an azerotrope, consisting of the benzene, a small amount of ethanol and all the remaining water in the solution. The azerotrope can then be boiled off as it evaporates at a lower temperature than ethanol. The remaining solution is 100% ethanol (SOLOMONS, 1988).

1.5.2. Acetaldehyde

Acetaldehyde is the direct precursor of ethanol in anaerobic respiration. Aldehydes are very similar to ketones in structure, consisting of a carboxyl group attached to a carbon chain. The name aldehyde means dehydrogenated alcohol. Occurring naturally as essential oils, aldehydes are partly responsible for the flavour of many fruits, as well as the odour of many plants. Benzaldehyde has the characteristic odour of cherries and almonds. Cinnamaldehyde, which occurs in oil of cinnamon, and the vanillin in vanilla beans are both aldehydes (ATKINS, 1989). Acetaldehyde, also called ethanal, is the simplest of the aldehydes (FIGURE 1.6), consisting of a carboxyl group attached to a methyl group (CH_3CHO). It is a liquid at room temperature, with a low boiling point of 21°C, and a melting point of –124°C. Acetaldehyde is largely used commercially for the synthesis of other organic compounds (GILLESPIE, HUMPHREYS, BAIRD and ROBINSON, 1989). To convert ethanol to acetaldehyde requires the removal of 2 hydrogens by oxidation. In humans, acetaldehyde is the product of ethanol oxidation in the liver, and its accumulation in the blood is one of the causes of hangovers (ATKINS, 1989). In the

ethanolic fermentation pathway, acetaldehyde is formed by the decarboxylation of pyruvate, catalysed by the enzyme pyruvate decarboxylase (PDC), producing CO_2 (KE, YAHIA, METEOS and KADER, 1994).

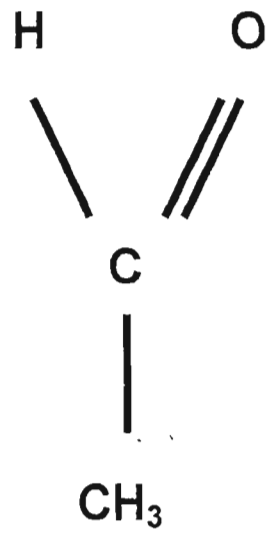


Figure 1.6. The chemical structure of acetaldehyde.

1.5.3. The role of ethanol and acetaldehyde in fruit ripening

Ethanol is formed in many fruits during the ripening process, together with volatile acids and esters (GERBER, 1896). Although levels are higher in fruits exposed to anaerobic conditions, ethanol has been observed in oranges stored in an oxygen-rich atmosphere (ONSLOW and BARKER, 1927), and can be isolated from tomatoes of any ripeness (GUSTAFSON, 1934; BIALE and YOUNG, 1962). The presence of ethanol is not always detected in tomato fruits (KELLY and SALTVEIT, 1988), and this may be due to losses from evaporation (SALTVEIT, 1989). Ethanol's precursor acetaldehyde is also found in natural plant tissues, although at very low levels. The ratio of ethanol to acetaldehyde is about 100 to 1 (THOMAS, 1925). This ratio can be increased to as high as 5:1 or even 2:1 by exposing the fruit to anaerobic conditions or a cyanide-rich environment (THOMAS, 1925; THOMAS and FIDLER, 1941). The naturally occurring

ethanol and acetaldehyde increase as senescence progresses in pears (JANES and FRENKEL, 1978) and tomatoes (GERBER, 1896). In yellow apples acetaldehyde levels may reach as high as 3% as they ripen (GUSTAFSEN, 1934). However, in very ripe fruit, acetaldehyde is converted to ethanol, which continues to accumulate (FIDLER, 1968).

Ethanol prevents the ripening of tomatoes, even in the presence of ethylene, although sometimes resulting in the fruit having a mottled appearance. Methanol and n-propanol were also tested. Methanol had no effect whatsoever, while n-propanol had an inhibiting effect slightly stronger than that of ethanol, with no negative effects on fruit appearance (KELLY and SALTVEIT, 1988, SALTVEIT and MENCARELLI, 1988; SALTVEIT, 1989). Ion leakage was increased as a result of n-propanol compared to ethanol, thereby damaging the fruit (SALTVEIT, 1989). Tomatoes are not the only fruit that are affected by ethanol treatment. When grapes were treated with ethanol, ripening was significantly inhibited (PESIS and MARINANSKY, 1992). As the ethanol concentration increased, so the ripening of tomato fruit decreased correspondingly. However, if ethanol levels within the tissues became too high, fruit ripening remained inhibited indefinitely (SALTVEIT and MENCARELLI, 1988).

It is well documented that ethanol disrupts the ethylene biosynthetic pathway in tomato fruit (JANES, CHIN and FRENKEL, 1978; SALTVEIT and MENCARELLI, 1988; BURDON, DORI, MARIANSKY and PESIS, 1994). When ethylene biosynthesis in ethanol-treated fruit was studied, ethanol appeared to prevent the conversion of ACC to ethylene, as no ethylene was formed, but ACC levels were 14 times higher (SALTVEIT and MENCARELLI, 1988). In grapes, although it did not prevent ethylene

formation, ethanol did prevent the normal climacteric respiration increase from occurring (PESIS and MARINANSKY, 1992). Ethanol also appeared to inhibit the action of ethylene (KELLY and SALTVEIT, 1988; SALTVEIT, 1989). The apparent prevention of ethylene action by ethanol may be due to it permeating into the lipid bi-layer of a selected membrane system associated with the ethylene action, most probably at the ethylene binding sites (SALTVEIT, 1989).

Like ethanol, acetaldehyde delays fruit ripening. The application of acetaldehyde to intact grape berries lead to increased CO_2 production, increased O_2 uptake and decreased ethylene evolution. This resulted in decreased acidity within the fruit, but no change in the total soluble solids content (PESIS and FRENKEL, 1989). When ACC was applied to untreated berries, ethylene production increased dramatically in untreated fruits, but in the presence of acetaldehyde the conversion of ACC to ethylene was inhibited (PESIS and MARINANSKY, 1992). An application of very low concentrations of acetaldehyde caused a delay in colour maturation of tomatoes (PESIS and MARINANSKY, 1993). The ripening of mango fruit (BURDON, DORI, LOMANIEC, MARINANSKY and PESIS, 1994) and kiwifruit (MENCARELLI, SAVARESE and SALTVEIT, 1991) are also inhibited by acetaldehyde treatment. Acetaldehyde appears to disrupt the ethylene biosynthetic pathway, affecting the action of both ACC synthase and ACC oxidase, and the accumulation of ACC (BEAULIEU, 1996; BEAULIEU and SALTVEIT, 1997; MENCARELLI, SAVARESE and SALTVEIT, 1991; PESIS and MARINANSKY, 1992). It appears however, that acetaldehyde has no effect on the action of ethylene, or the plant's sensitivity to it.

Acetaldehyde can also promote fruit ripening. In pears, as levels of ethanol and

acetaldehyde increased, so did the softness of the fruit, and ethylene production (JANES and FRENKEL, 1978). This softening of the pear was not directly due to ethylene production, as even after the application of an ethylene inhibitor, as well as acetaldehyde, fruit softening still occurred. No softening occurred in the absence of acetaldehyde (JANES and FRENKEL, 1978). Exogenously applied acetaldehyde resulted in an increased ripening rate and the stimulation of ethylene production (JANES and FRENKEL, 1978). The levels of acetaldehyde required to promote ethylene biosynthesis were low, much lower than the levels that inhibit ethylene production. The mechanism of the ethylene promotion is not understood, although it is known to occur independently of any ethylene action (JANES and FRENKEL, 1978).

The relationship between ethanol and acetaldehyde during fruit ripening is not clearly understood. The two chemicals are formed by the same process, are both two carbon compounds, and have very similar effects on the inhibition of fruit ripening and ethylene formation. When an inhibitor of alcohol dehydrogenase was added to ethanol-treated flowers, thereby preventing the conversion of ethanol to acetaldehyde, no inhibition of fruit ripening occurred. Acetaldehyde was named as the causal agent of ethanol-induced inhibition of tomato ripening (BEAULIEU, 1996; BEAULIEU, PIESER and SALTVEIT, 1997; BEAULIEU and SALTVEIT, 1997). The details and implications of this discovery will be discussed further in Chapter 4.

1.5.4. Ethanol and cut carnation flower senescence

Ethanol is a natural component of senescing cut carnation flowers, and as mentioned in Section 1.4.2.1, it is a natural post-harvest treatment. Ethanol was first discovered to extend the vase-life of cut carnation flowers in 1980, when it was reported

that a solution of 7.5% ethanol increased longevity by 200% (HEINS, 1980). Other alcohols were tested, but this effect was limited to ethanol treatment (HEINS and BLAKELY, 1980; WU, ZACHARIAS, SALTVEIT and REID, 1992).

Ethanol completely prevented ethylene production, and reduced the related burst in CO_2 production by 60% in cut carnation flowers (HEINS and BLAKELY, 1980; WU, ZACHARIAS, SALTVEIT and REID, 1992). Ethanol was shown to inhibit the conversion of ACC to ethylene (HEINS and BLAKELY, 1980), and reported to inhibit the action of ACC synthase as well as the formation of ACC (WU, ZACHARIAS, SALTVEIT and REID, 1992). The effectiveness of ethanol at delaying senescence was only observed if ethanol was applied before the onset of climacteric respiration and autocatalytic ethylene production (HEINS, 1980). When petal discs were tested, small amounts of ethanol were required to prevent ethylene production before the onset of senescence. Much higher concentrations of ethanol were required after senescence had begun. These concentrations were phytotoxic however, and the effect on ethylene inhibition may be due to another mechanism (HEINS and BLAKELY, 1980). Apart from initial ethylene production, autocatalytic production was also reduced in ethanol-treated flowers, indicating that ethanol has an effect on ethylene action and the sensitivity of the plants to ethylene. This effect is a double reciprocal plot, meaning that the inhibition of ethylene action is non-competitive (WU, ZACHARIAS, SALTVEIT and REID). This is in direct conflict with a previous report, where ethanol was found to have no effect against exogenously applied ethylene (HEINS and BLAKELY, 1980). A number of changes are observable in ethanol-treated flowers. The most visible change is the inhibition of petal wilting, which is replaced by petal browning (HEINS, 1980). The most important morphological change however, is the inhibition of ovary development (HEINS

and BLAKELY, 1980).

It is clear that ethanol is a potent inhibitor of flower senescence, and a suitable post-harvest treatment. However, since its initial discovery in 1980, only one research paper has been published on the effect of ethanol on cut carnation flowers. The main reason for the apparent lack of interest in ethanol as a post-harvest treatment was the concurrent discovery of STS. Unlike ethanol, STS is highly effective against exogenously applied ethylene. It is also quick and easy to apply, making it an ideal commercial post-harvest treatment. There seemed little point in researching the effect of ethanol any further. However, there is now a need to find a replacement treatment for STS. Silver is a heavy metal, and therefore an environmental pollutant. The European Union has banned the importation of any produce containing heavy metals, and this has had a huge impact on the business of cut flower growers. Commerce aside, during these environmentally conscious times, it is necessary to find a more natural, environmentally friendly, post-harvest treatment. Ethanol is a natural compound, and if it were used as a commercial post-harvest treatment it would thereby solve both problems. However, before it can be used as a treatment certain factors remain to be determined, such as its suitability as a post-harvest treatment, and the reason for the physiological responses seen during ethanol-effected senescence.

CHAPTER 2

THE POTENTIAL OF ETHANOL AS A POST-HARVEST TREATMENT FOR CUT CARNATION FLOWERS

2.1. Introduction

Investigation into the use of ethanol as a post-harvest treatment began in 1978. It was reported to Professor RD Heins that a certain compound extended the vase life of cut carnation flowers greatly. He decided to test these results himself, and observed that the compound in question was not soluble in water. Instead it had to be dissolved in alcohol. As a control treatment Professor Heins treated flowers with the same concentration of alcohol in which he had dissolved the compound. It transpired that it was in fact the small amount of alcohol used as a solvent, and not the compound itself, that resulted in the increased longevity that was observed (RD HEINS, personal communication, 1996).

Heins then conducted a number of longevity trials using a low concentration of ethanol, and found that the resultant vase life varied greatly from one experiment to the other. For example flowers treated with three percent ethanol varied in vase life from eight days to twenty one days between experiments. A major reason for the problem was loss of ethanol through evaporation. However, even after this problem was eliminated, variation still occurred. Ethanol solutions were changed at various times

throughout the vase life of the flowers (i.e. every day, every second day and so forth) in an attempt to control the variation in vase life. The keeping life of the flowers decreased, as the frequency of change decreased. Similarly when ethanol was applied to flowers for the first time on the day of harvest and the second day of the experiment, the vase life increased. However, when treatment only commenced on fourth, eighth and tenth day of experimentation, flower senescence was simultaneous with the senescence of the control flowers. This indicated that ethanol must be applied to the flower fairly early in the vase life in order to have a significant effect (HEINS, 1980). It was also observed that the beneficial effects of ethanol were only seen when ethanol was kept in constant supply (WU, ZACHARIAS, SALTVEIT and REID, 1992).

A range of concentrations was tested, from 0.5% to 26%, with the resultant improvement in the keeping life increasing from a minimum 46% for 26% ethanol, to a maximum 232% for 8% ethanol (HEINS, 1980). There was a problem with the extremely high concentrations of ethanol tested, for as well as not extending the vase life of the flowers, treatments above 20% also caused stem toppling to occur, thereby ending the vase life of the flowers (HEINS, 1980; WU, ZACHARIAS, SALTVEIT and REID, 1992). Ethanol at concentrations of 10% and higher appeared to be phytotoxic, as petal tissue became soaked and leaked pigments (HEINS and BLAKELY, 1980). Visually, flowers treated with ethanol differed from control flowers in that the typical "in-rolling" of the petals did not occur. Instead, individual petals developed necrotic tips after 10 - 11 days of treatment, and several days later the entire petal dried out. Certain petals remained turgid while adjacent petals completely dried out (HEINS, 1980). A variety of pulse treatments were applied to the flowers. The length of the pulse, as well as the concentration of ethanol applied, were varied, but they were

ineffective at delaying the senescence of the flowers (HEINS and BLAKELY, 1980; WU, ZACHARIAS, SALTVEIT and REID, 1992).

Soon after the discovery of ethanol's activity at extending the vase life of cut carnation flowers, the effect of silver thiosulphate (STS) was discovered (STABY, CUNNINGHAM, EISENBERG, BRIDGEN and KELLY, 1978; SPEAR and GLADON, 1982; CHUNG, LEE and KIM, 1986; COOK and VAN STADEN, 1987). Because of the powerful effect of STS on the vase life of the flowers, and the great economical benefits of its use, little further research has been done into the potential of the use of ethanol as a commercial post-harvest treatment. With the need to find an environmentally friendly treatment to replace the commercially used heavy metal STS, the time is right to research the potential of ethanol as an efficient post-harvest treatment. The aim of the research conducted in this Chapter was to answer the question: Is ethanol suitable to be used as a post-harvest treatment for cut carnation flowers?

2.2. Materials and methods

2.2.1. Plant material

Dianthus caryophyllus L. (cv. White Sim) flowers were used for these, and all further experiments. Flowers were obtained from Floricadia, a commercial grower in Heidelberg, Gauteng, South Africa. The flowers were picked at, or as close as possible to, the stage directly before commercial usage (FIGURE 2.1). The flowers were transported to Pietermaritzburg by air, arriving late in the afternoon of the day that

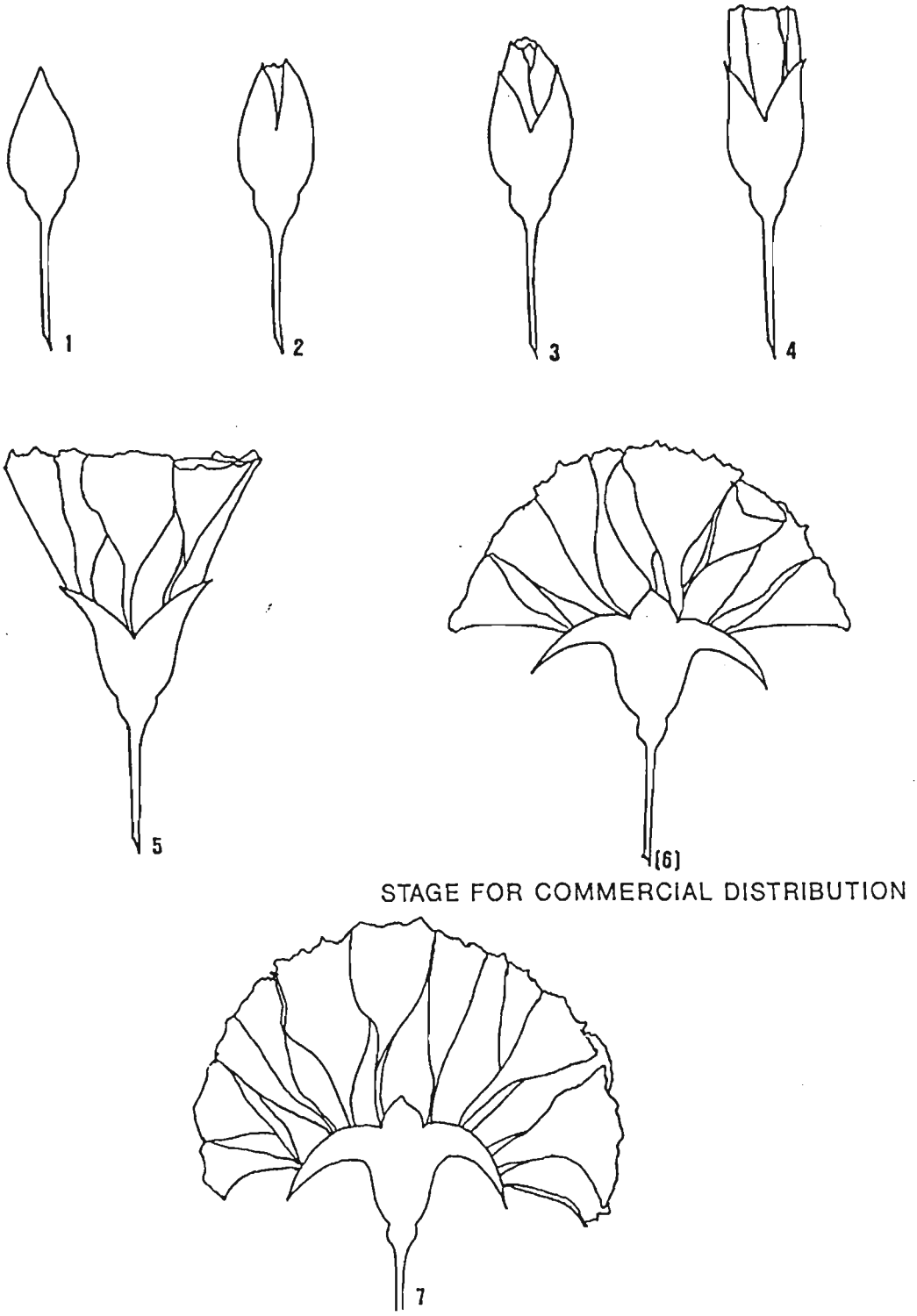


Figure 2.1. Stages of carnation flower development, from closed bud to fully reflexed petals. Stage 6 is the stage at which the flowers are ready for commercial distribution (After UPFOLD, 1992).

they had been harvested. Upon unpacking, the flowers were placed in distilled water for a period of between 16 and 24 h in order to acclimatise to laboratory conditions after transportation, and also to avoid embolism formation. After this, the stems were trimmed to 10 cm, except for the experiment in which the effect of stem length on ethanol induced longevity was tested. For this experiment stems were cut to 2, 5, 10, 20 and 30 cm, with 10 flowers in each treatment.

2.2.2. Holding the flowers

Flowers were kept in an air-conditioned laboratory, with a standard temperature of 22°C and an average of 10 h fluorescent light exposure per day. These conditions were used, rather than a controlled environment, as they came closer to imitating the typical conditions flowers would be exposed to in the average consumer's home. Each individual flower was placed in a 500 mℓ glass jar. These jars contained the relevant holding solutions. A tin foil cap covered the entrance.. This was not only to prevent excess evaporation from the solution, but also to support the flowers, allowing the base of the stems to hang free within the solution, optimising solution uptake.

2.2.3. Ethanol treatments

All ethanol solutions were prepared using 95% ethanol and were diluted with distilled water. These solutions were replaced every two days in order to minimise any evaporation effects. For all experiments, a distilled water control was provided. For longevity trails that involved continuous treatment, 100 mℓ of each concentration of ethanolic solutions (from 0 to 20%) were placed in the glass jars

described in Section 2.2.2. If a longevity trial involved a delay in ethanol application, or a limit on the length of application, distilled water was used as a substitute holding solution when necessary. Pulse treatments were placed in pill vials, and these were then placed inside the glass jars. The length of the pulse treatments varied, and included a 1, 2, 4 and 24 h pulse. After this time had elapsed, the pill vial was removed and replaced with 100 mℓ distilled water.

2.2.4. Statistical analysis

A minimum of 10 flowers were used for each treatment. In all instances where variables were recorded, at least 3 repetitions were used for each of the treatments, and all experiments were repeated a minimum of three times. Applicable data was analysed using a multiple range test, and a one way analysis of variance (ANOVA) at a confidence level of 95%.

2.2.5. S50 and S100 readings

It became evident during this study that an expression of the degree of senescence of each group of flowers was necessary. This is done using S50 and S100 values. S50 values indicate the time period that had elapsed between the onset of treatment, and half of the flowers being at the end of their vase life. In a few experiments S100 readings were taken, indicating the time elapsed between onset of treatment and the vase life of all the flowers being considered terminated. This value was used for situations in which one or more of the treatments senesced exceedingly fast. Various parameters had to be taken into account when considering flowers to be at either S50 or S100. These included the diameter of the floral head,

the degree of both petal in-rolling and petal browning, as well as any unusual responses that would make the flower undesirable to consumers, such as stem bending or the petals becoming water logged. Examples of these situations are presented in PLATE 2.1.

2.2.6. Ethylene determination

Ethylene production was determined by enclosing flowers in 1ℓ glass jars for a period of 1 h, after which a 1mℓ gas sample was extracted. This sample was then subjected to gas chromatography on a Varian 3300 gas chromatograph, fitted with a photon ionization detector. The column was a 1 m by 3.2 mm aluminum F-1 column, packed with 80-100 poropak SIS. Operating temperatures were column 80°C, injector port 100°C and detector 100°C. Hydrogen was used as the carrier gas, at a flow rate of 60mℓ.min^{-1}. A standard curve for ethylene concentration was constructed (FIGURE 2.2). A representative chromatogram is available in APPENDIX A.

2.2.7. Exogenously applied ethylene

Flower senescence can be advanced by supplementing the natural levels of ethylene in the organ, resulting in both senescence-like responses, and the *de novo* synthesis of ethylene. The ethylene supplement used in these experiments was 2-chloroethyl phosphonic acid, commercially known as ethephon or ethrel ®. When absorbed by living tissues, ethrel is converted to ethylene (WARNER and LEOPOLD, 1969). Flowers treated with ethrel were kept separate from control flowers by distance.

Plate 2.1. Petal in-rolling (A), petal wilting (B) and petal browning (C) seen in ethanol-treated and control flowers.

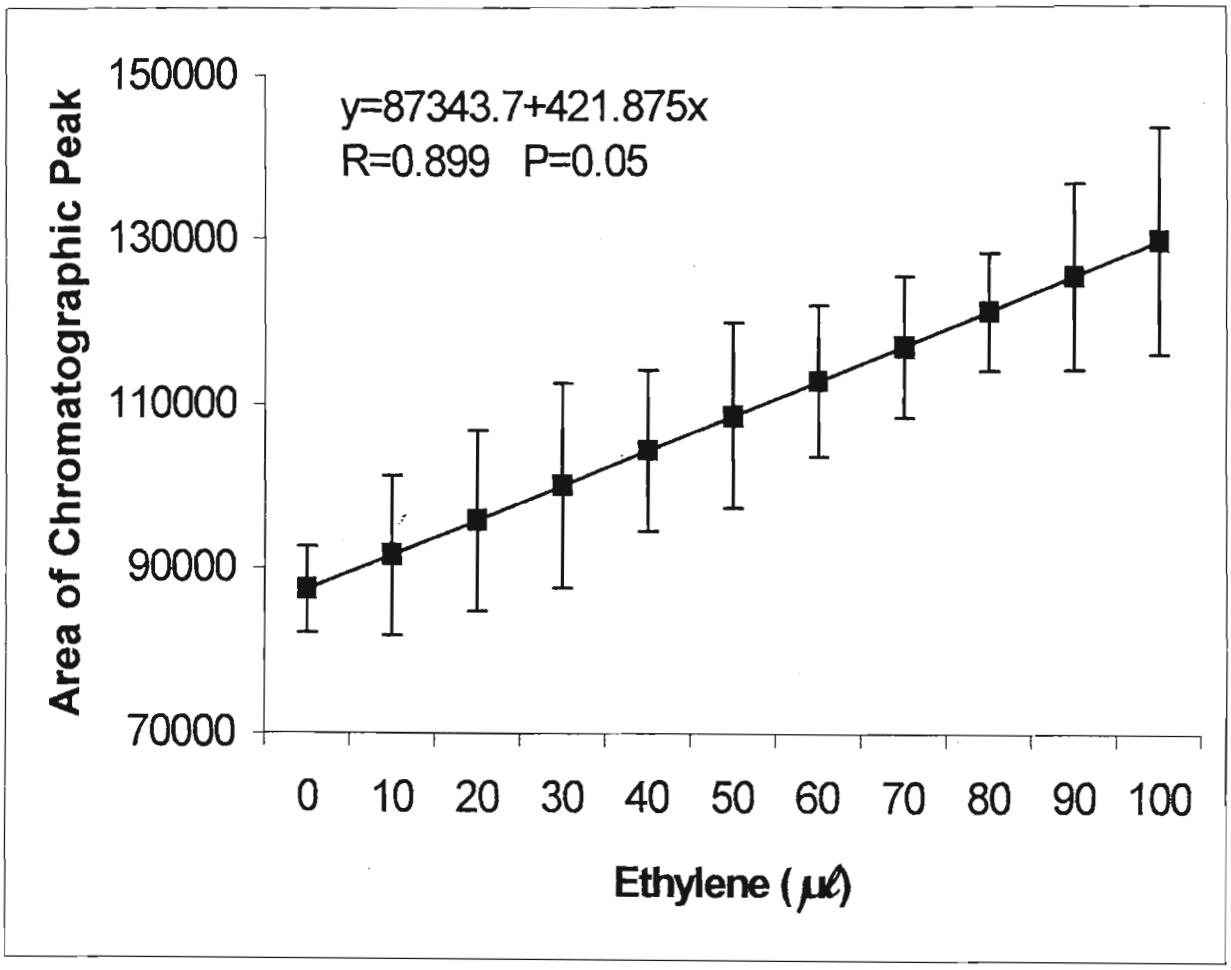


Figure 2.2. Standard curve for ethylene determination by chromatography. Bars indicate standard errors.

2.3. Results

During the original research conducted into the effect of ethanol treatment on cut carnation flowers, it was found that the most effective concentration of ethanol varied from one experiment to another (HEINS, 1980). However, in order to use ethanol as a post-harvest treatment, a concentration of ethanol must be selected that is the most efficient. Seven concentrations of ethanol were applied to flowers (from 0% through to 20%), on five different occasions. The results are represented in TABLE 2.1.

Table 2.1. Longevity results (days) for seven concentration of ethanol. Five repetitions are shown. The standard deviation (std dev) about the mean is indicated.

	Ethanol Concentration (%)						
Repetition	**0**	**1**	**3**	**5**	**8**	**10**	**20**
1	6	14	15	12	10	9	8
2	8	14	17	15	11	10	8
3	5	10	10	11	10	8	5
4	12	18	22	21	16	12	12
5	7	10	11	12	9	8	8
Mean	**7.6**	**13.2**	**15**	**14.6**	**11.2**	**9.4**	**8.2**
Std dev	**±2.7**	**±3.35**	**±4.85**	**±3.78**	**±2.77**	**±1.67**	**±2.49**

The efficiency of each concentration did indeed vary, with the concentration that gave the greatest vase life increase alternating between 3% and 5% ethanol. If one considers the mean vase life of the five repetitions, 3% ethanol treatment resulted in the longest vase life, 197% longer than the water control. Despite the variations within

the results, a number of clear patterns emerged. Low concentrations of ethanol (1 - 5%) resulted in the greatest longevity increases, and the flowers maintained a healthy appearance throughout their vase life (PLATE 2.2). If the applied concentration was increased, to levels of 8% or higher, negative effects became evident, such as extensive discolouration, severe petal burn and water-logging of the petals. Low concentrations of ethanol (1 – 3%) resulted in the greatest longevity increases. The flowers were more sensitive to increased concentrations of ethanol. For example, in the first repetition of the longevity trial (TABLE 2.1), when 1% ethanol was almost as effective as 3% ethanol, discolouration was observed in the stem and calyx of flowers treated with 5% ethanol. On the other hand, in the third repetition, when 5% ethanol was the most effective concentration, no negative effects were observed in flowers treated with less than 10% ethanol.

The most effective concentration of ethanol was not the only variation seen during the longevity trial. The length of the resultant vase life of the flowers also varied greatly, concurring with the results reported by HEINS (1980). In one case flowers treated with 3% ethanol lived for 22 days after harvest. In another case, however, the maximum-recorded vase life of treated flowers was 11 days. Both of these situations seem to indicate a change in the flower's sensitivity to ethanol treatment from experiment to experiment. The reason for the variation in both flower longevity and concentration effectiveness is probably due to the physiological state of the flower at the onset of treatment (HEINS, 1980). Factors that would effect the physiological state of the flowers, and therefore their sensitivity to ethanol, include the age of the flowers at harvest, water stress that might have occurred during transportation and harvesting injuries. As the flowers used in these experiments were grown commercially, it was

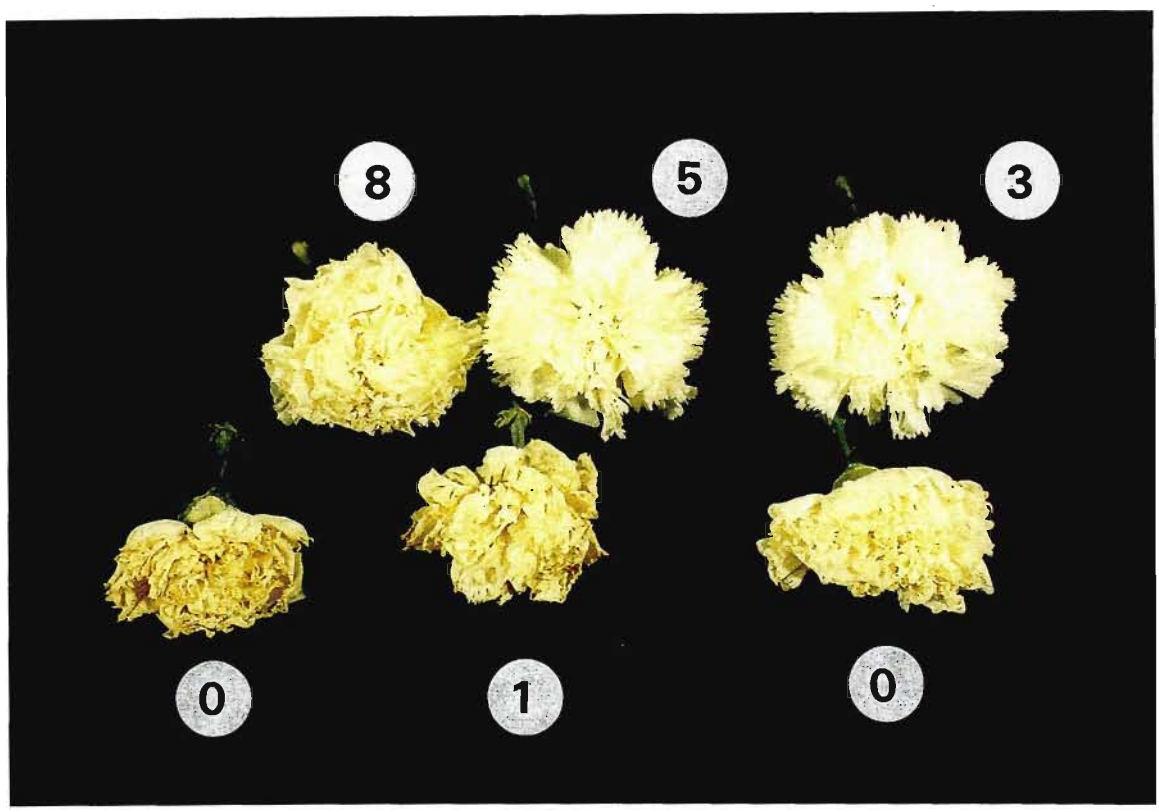


Plate 2.2. Appearance of ethanol-treated carnation flowers 10 days after harvest. Text boxes indicate the concentration of ethanol applied. Note that ethanol-treated flowers still appear healthy.

impossible to control the harvesting of the flowers. Experiments had to be designed to allow for variation in the flower's responsiveness to ethanol. Therefore 3% ethanol was used as the standard holding solution for subsequent experiments. Not only was it the most effective overall concentration, but it falls into the buffer zone of concentrations. It is low enough to ensure that no negative effects occur, but high enough to ensure longevity increases in the flowers, regardless of the sensitivity of the particular batch of flowers.

The commercially used post-harvest treatment, STS, is applied to carnation flowers as a 15 min pulse, and this extends the vase life of the flowers by some 250%. Ethanol was applied to carnation flowers, at a concentration of 3%, as a pulse treatment for various time periods (FIGURE 2.3). None of the pulse treatments extended the vase life of flowers significantly, verifying the results of WU, ZACHARIAS, SALTVEIT and REID (1992). A higher concentration pulse was also attempted (20%), but this did not increase the vase life of the flowers either. In fact it resulted in phytotoxicity within the flower, reducing the vase life of the flower compared with the water controls.

If it cannot be applied as a pulse, an efficient post-harvest treatment needs to have the shortest possible application time. When 3% ethanol was applied to flowers for various lengths of time, ranging from continuous application, to a one day pulse treatment (FIGURE 2.4), the resultant vase life of the flowers was shown to be directly dependent on the length of ethanol application. A linear response was observed, indicating, that the longer the time period of ethanol application, the longer the vase life of the flowers. As soon as flowers were removed from the presence of ethanol, normal

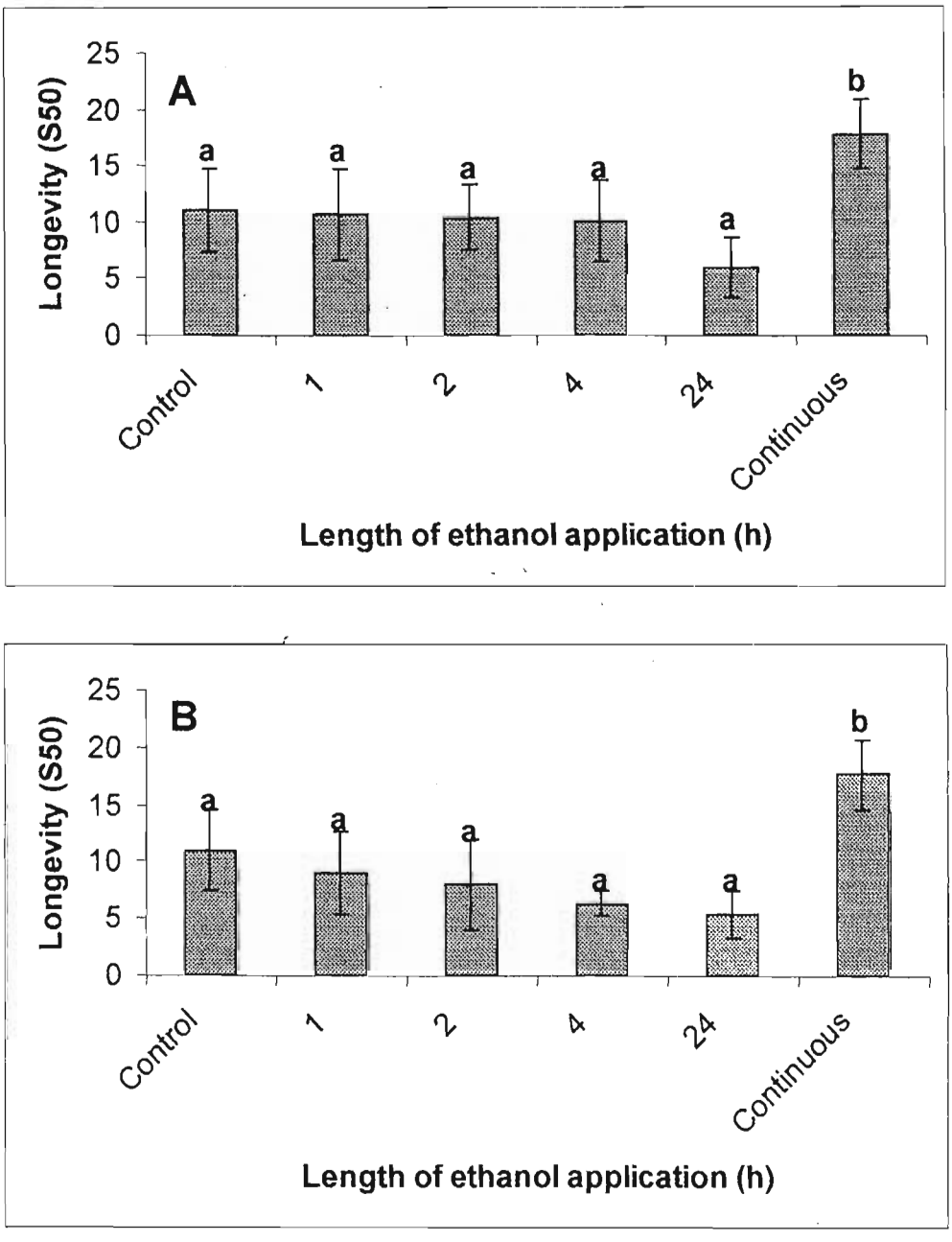


Figure 2.3. The effect of a pulse treatment of 3% ethanol (A) and 20% ethanol (B) on the longevity of cut carnation flowers. Bars indicate standard errors within the treatments. Treatments with the same letter are not significantly different from one another.

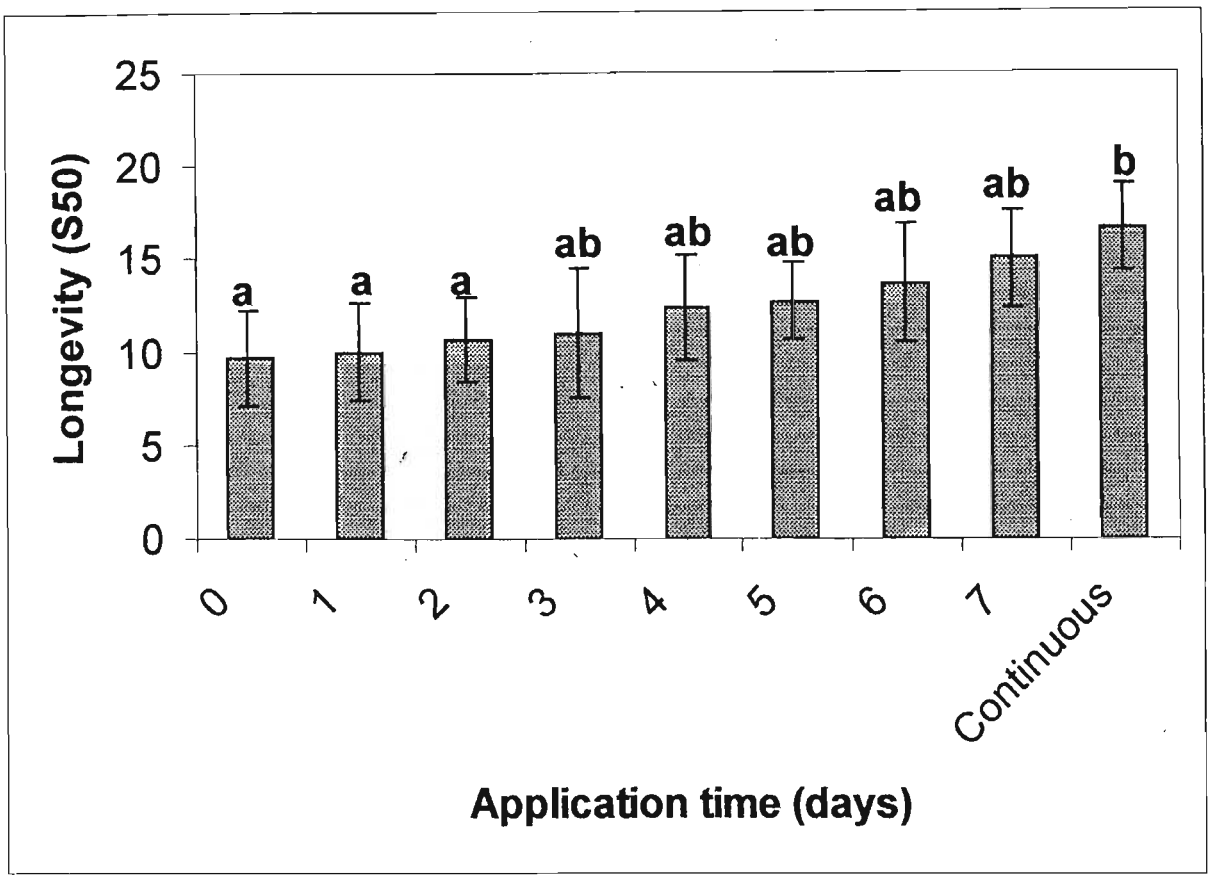


Figure 2.4. The effect of the length of ethanol application on the resultant vase life of cut carnation flowers. Bars indicate standard errors within the treatments. Treatments with the same letter are not significantly different from one another.

senescence occurred. The most efficient treatment by far was the continuous 3% ethanol holding solution. One and two day treatments resulted in no significant vase life increase compared to the distilled water control. There was an indication that ethanol arrested, but did not permanently inhibit the senescence process, as senescence continued at the same rate as the water control when ethanol treatment was removed. This validates the observation made by WU, ZACHARIAS, SALTVEIT and REID (1992), who stated that the benefits of ethanol treatment are only achieved when ethanol is in constant supply.

Ethylene production is an important factor in carnation flower senescence. The effect of ethanol treatment on ethylene production was determined for a number of ethanol treatments (FIGURE 2.5). It is apparent that ethanol treatment almost completely prevented ethylene production, and inhibited the autocatalytic rise in ethylene production seen in normally senescing climacteric flowers. These results concurred with those reported by previous workers (HEINS, 1980; HEINS and BLAKELY, 1980; WU, ZACHARIAS, SALTVEIT and REID, 1992). There was no significant ethanol concentration effect observed, with even low levels of ethanol preventing ethylene formation. An observable trend was that the greater the applied concentration of ethanol, the less ethylene was produced. The mechanism of the interruption of ethylene biosynthesis by an ethanol holding solution will be discussed in more detail in Chapter 3.

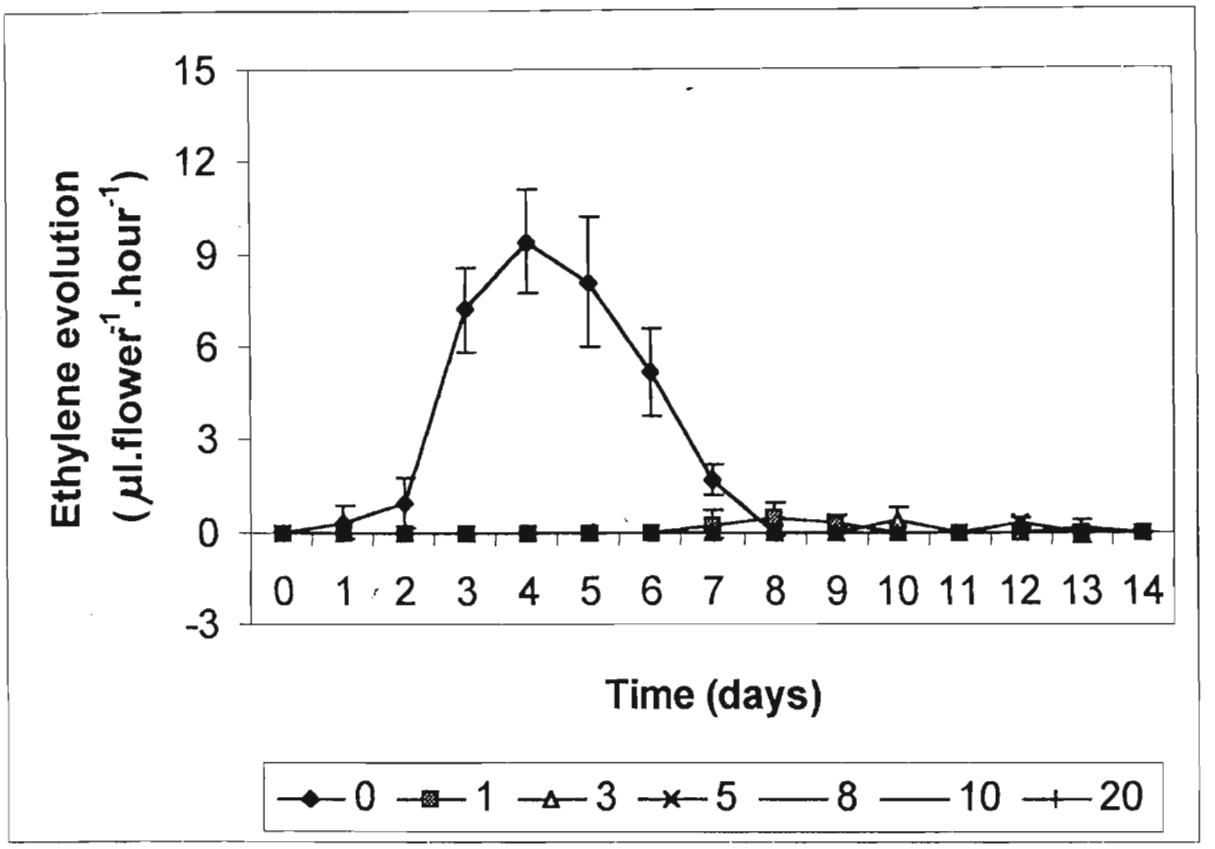


Figure 2.5. The effect of various concentrations of ethanol on ethylene production by cut carnation flowers. Bars indicate standard error within the treatments.

While the inhibition of ethylene production by a post-harvest treatment is important, if the action of ethylene is not blocked it will still hold many problems for the floriculturist. Even when endogenous ethylene production is completely inhibited, if the action of exogenous ethylene is not prevented, protein production and resultant senescence will still occur. The exposure of ethanol-treated flowers to a source of exogenous ethylene, in the form of ethrel ®, resulted in a reduction in the vase life (FIGURE 2.6). Flowers treated with ethanol, which were not exposed to ethylene, lived significantly longer than those that were. However, the ethanol-treated flowers did live longer than control flowers in the presence of ethylene, indicating some disruption in exogenous ethylene action. The concentration of ethanol applied had no significant effect on the blockage of ethylene action.

If ethanol treatment prevents the biosynthesis of ethylene, and resultant flower senescence, it is necessary to establish when ethanol treatment must begin. Using a 3% holding solution, the onset of ethanol treatment was delayed for various times (FIGURE 2.7). A linear response was observed, whereby the longer the delay in ethanol application, the shorter the resultant increases in vase life. Ethylene production by the control flowers was recorded during this experiment, and the climacteric ethylene production was found to occur on the fourth day of the experiment. The results show that if ethanol was applied to the flowers before climacteric ethylene production had occurred, there was a significant increase in the vase life of the flowers compared to the water control. If ethanol was applied after the ethylene climacteric there was not a significant increase in vase life. This is similar to the results of HEINS (1980).

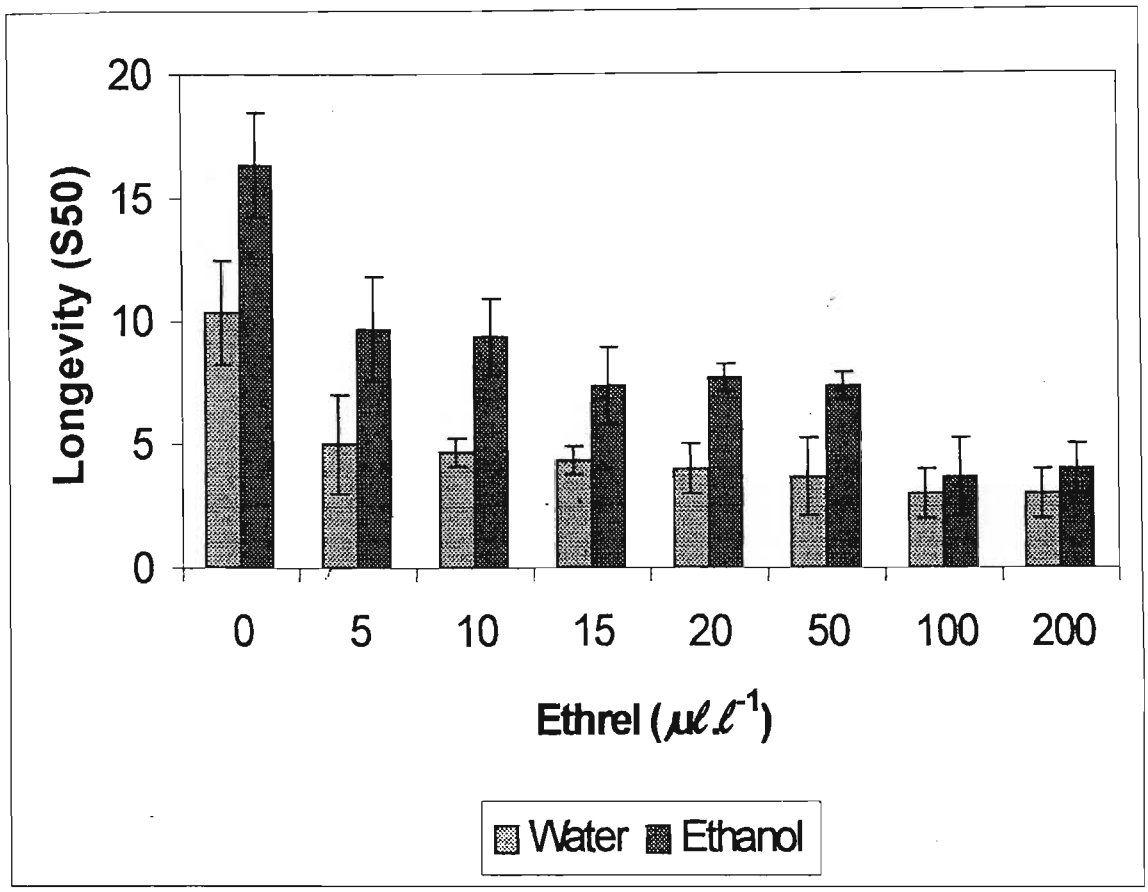


Figure 2.6. The effect of ethrel ® on the vase life of cut carnations. Bars indicate standard errors within the treatments.

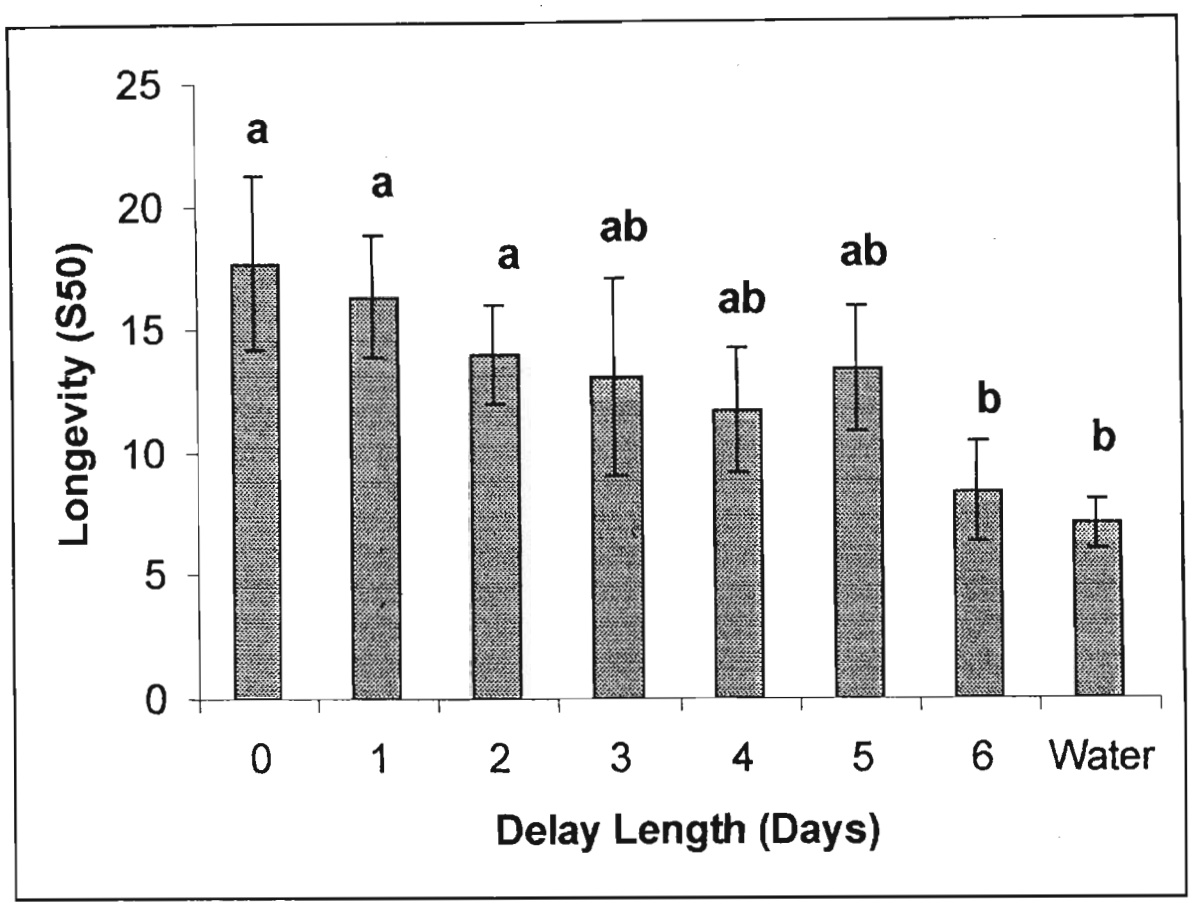


Figure 2.7. The effect of a delay in ethanol application on the longevity of cut carnation flowers. Bars indicate standard errors within the treatments. Treatments with the same letter are not significantly different from one another.

All the flowers used in these experiments had their stems trimmed to 10 cm, in order to maintain uniformity. However, in normal circumstances the stems would be much longer. In order to determine whether the length of the flower stem has any effect on ethanol treatment, flowers with stems varying in length from 2 cm to 30 cm were treated with 3% ethanol (FIGURE 2.8 and PLATE 2.3). The flowers with longer stems survived significantly longer than flowers with short stems. A linear relationship between stem length and longevity appears to exist.

2.4. Discussion

There is a discrepancy in the literature regarding the effectiveness of ethanol against externally applied ethylene. It was initially reported that ethanol has no effect against external ethylene (HEINS and BLAKELY, 1980). However, in a later report it was suggested that ethanol inhibits ethylene action, in a non-competitive manner (WU, ZACHARIAS, SALTVEIT and REID, 1992). In STS-treated flowers, silver ions actively compete with ethylene for the binding sites. However, it has been suggested that there is no competition in ethanol-treated flowers for the ethylene binding sites. If ethylene binding was inhibited, senescence would remain suspended, as in the case of STS-treated flowers. If no prevention of ethylene action occurred, the external ethylene would bind to the membrane bound binding sites, leading to two things. Firstly, it would cause the production of proteins and enzymes which lead to senescence-like responses. Secondly, it will result in the *de novo* synthesis of more ethylene, resulting in climacteric ethylene production. Current results indicated that in ethanol-treated flowers exposed to external ethylene, senescence symptoms were manifested. This suggests that ethylene binding had taken place, but no resultant ethylene climacteric

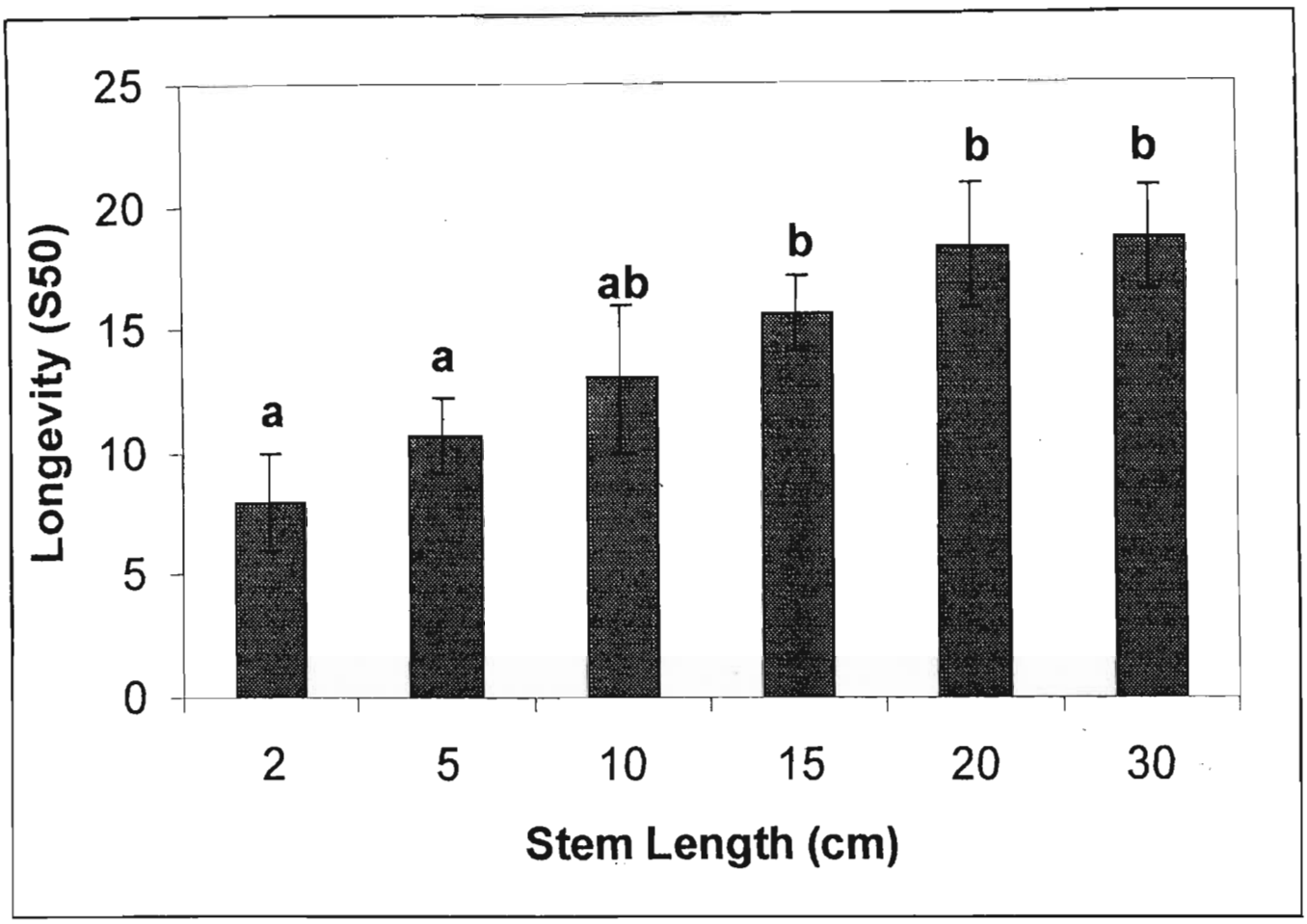


Figure 2.8. The effect of stem length on the longevity of ethanol-treated cut carnation flowers. Bars indicate standard errors within the treatments. Treatments with the same letter are not significantly different from one another.

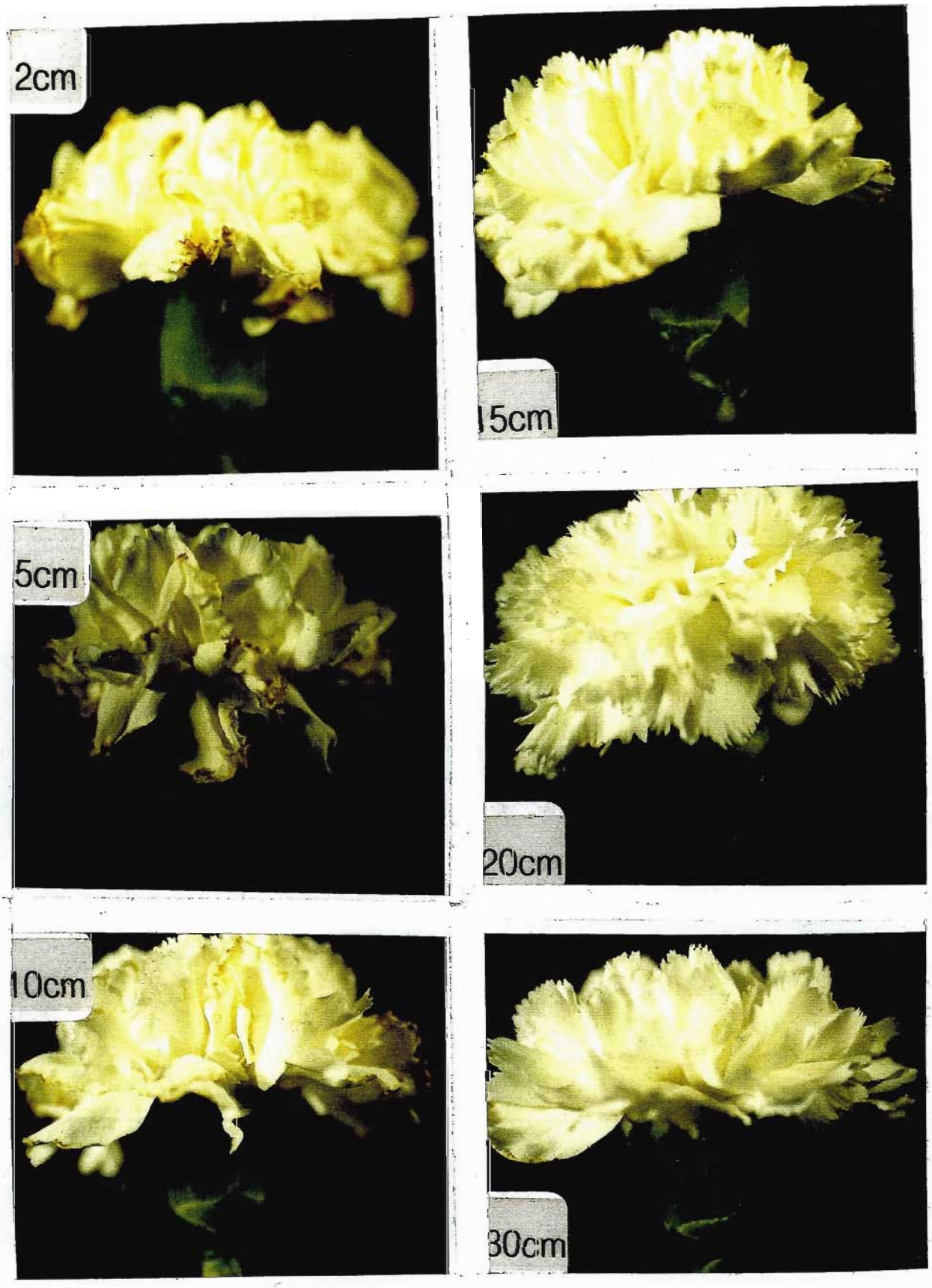


Plate 2.3. The effect of a variation in stem length of flowers treated with 3% ethanol (Day 9). Text boxes indicate the length of the stems of the flowers.

rise occurred after exogenous ethylene treatment. It has already been suggested (HEINS and BLAKELY, 1980) and confirmed that ethanol inhibits ethylene formation, as little to no ethylene is formed in ethanol-treated flowers. Therefore it is quite possible that, rather than any actual inhibition of the action of external ethylene, the apparent minor inhibition of ethylene action is caused by the blockage of further ethylene synthesis,

Results from longevity trails involving a delay in ethanol application suggests that ethanol must be applied before autocatalytic ethylene production has begun, otherwise ethanol is not able to prevent the climacteric. This concurs with the suggestion that ethanol does not inhibit ethylene action significantly, but rather prevents ethylene formation within the system. However, if large scale ethylene biosynthesis has already begun, the effect of ethanol is not so pronounced.

A possible reason for the existence of the relationship between stem length and vase life could be because the ethanol must now travel significantly further in order to reach the floral head. The on-set of the negative effects of ethanol were also delayed, such as petal browning or water-logging. On the other-hand, ethanol treatment might then immediately prevent the formation of wound-induced ethylene by the stems and other organs.

The question posed at the beginning of this Chapter was whether ethanol would make a suitable post-harvest treatment for cut carnation flowers. The characteristics of an efficient post-harvest treatment include simple application (such as a pulse treatment), cost effectiveness, ease of transportation, and of course, effectiveness at

extending the vase life of the flowers. It is also vital that the compound be user friendly, (and not cause ill health, such as 2,5 Norbornadiene), and that it is environmentally acceptable (unlike STS). Ethanol does indeed extend the vase life of cut carnation flowers significantly, and it is a natural and innocuous compound. It does fail many of the other criteria though. Firstly, it cannot be applied as a pulse treatment, or even as a holding solution for one or two days only. Ethanol must be applied to the flowers as soon as possible, for as long as possible. This fact alone makes it unsuitable as a post-harvest treatment. It is impractical to have a post-harvest treatment for which treatment must begin immediately after picking, and continue throughout all stages of the flower's vase life, even after reaching its final destination, the consumer. To make matters worse, if at any point the flower is removed from the ethanol solution, normal senescence ensues. Another problem is transportation, as not only would the flowers have to be transported in buckets containing ethanol, but they would also have to be in the presence of ethylene scrubbers. This is because, even though it prevents ethylene biosynthesis, ethanol does not inhibit the effect of exogenous ethylene. Even the smallest amount of ethylene present during transport could result in senescence of the crop. The biggest problem with ethanol as a post-harvest treatment however, is the inconsistency of its effectiveness and the flowers resultant vase life. Although ethanol treatment does increase the vase life of cut carnation flowers significantly, the degree of increase, as well as the most effective concentration, does not remain constant from one group of flowers to the next. This inconsistency would be unacceptable to consumers.

Although an ethanol holding solution is not suitable for commercial use, this does not mean that there is not more research to do. The use of skills such as genetic

manipulation and gene silencing could result in the negative effects of ethanol treatment being removed. It is also possible that ethanol, in conjunction with one or more natural compounds, may prove to be the ideal post-harvest treatment. However, at this point it must be concluded that ethanol treatment alone will not replace STS as the preferred commercially used post-harvest treatment.

CHAPTER 3

THE PHYSIOLOGICAL RESPONSE OF CUT CARNATION FLOWERS TO ETHANOL AS A POST-HARVEST TREATMENT

3.1. Introduction

Little research has been conducted into the physiological responses of cut carnation flowers to ethanol. The literature indicates that ethanol plays some role in disrupting the production of ethylene, normal assimilate movement within the tissues, the respiration rate of the flowers and possibly the action of ethylene.

In normally senescing flowers the petals roll inwards and become less turgid during senescence. When ethanol was applied to cut carnation flowers however, it resulted in the petals appearing necrotic at the tips after 11-12 days, and eventually drying out entirely (HEINS, 1980). This process is described as slow dying from the tips (WU, ZACHARIAS, SALTVEIT and REID, 1992), and the effect named burning or browning (HEINS, 1980). The reaction of the petals to ethanol was not uniform, with some petals completely burnt, while others still appeared healthy and turgid. It was proposed that this occurred due to a certain ethanol level being reached within the individual petals. Before this level is reached, no burning is thought to occur (HEINS, 1980). There was a marked response in ovaries of ethanol-treated flowers. During normal senescence the chlorophyll content of the ovary increases, due to well-developed chloroplasts induced by ethylene formation (COOK and VAN

STADEN, 1983). It also gains a large amount of weight, and increases in diameter. The ovaries of the ethanol-treated flowers neither gained weight nor increased in diameter (HEINS and BLAKELY, 1980), and no chloroplast development was recorded (COOK and VAN STADEN, 1983). The effect of ethanol on the fresh weight of the entire flower was recorded. As the senescence process progressed, there was a huge decrease in the weight of the control flowers. Ethanol treatment originally resulted in a decrease in fresh weight compared to flowers held in water, but no further weight loss was recorded. At the end of the experiment the ethanol-treated flowers weighed more than the control flowers (WU, ZACHARIAS, SALTVEIT and REID 1992).

The application of ethanol to cut carnation flowers resulted in a large decrease in, or even complete inhibition of, the climacteric burst in ethylene associated with senescence. This was due to the total prevention of ethylene formation by ethanol. When a low concentration of ethanol was applied to flowers, a very small amount of ethylene was detected. At higher concentrations, no ethylene production was reported. It was proposed that ethanol prevented ethylene formation by inhibiting the synthesis of ACC oxidase, but not the action of the enzyme once it was formed (HEINS and BLAKELY, 1980). When flowers were placed in a solution of ACC, in the presence of ethanol, the conversion of ACC to ethylene was prevented, indicating that ACC oxidase activity was inhibited (HEINS and BLAKELY, 1980; WU, ZACHARIAS, SALTVEIT and REID, 1992). A reduction in the levels of ACC was also observed, indicating that ethanol not only affected the action of ACC oxidase, but also the formation of ACC, the action of ACC synthase or the storage of ACC as MACC (WU, ZACHARIAS, SALTVEIT and REID, 1992).

Ethanol blocks the formation of ethylene in non-climacteric tissues. If the tissue was already climacteric, higher concentrations were required to inhibit ethylene formation (HEINS and BLAKELY, 1980). Climacteric ethylene synthesis is usually concomitant with a rise in the respiration rate. Ethanol treatment depressed the respiration rate of the flowers by up to 60% (HEINS and BLAKELY, 1980). When three different concentrations of ethanol were tested, it was observed that the higher the ethanol concentration, the greater the reduction in the respiration rate. However, the timing of this reduction in respiration was not changed, and the partial suppression of the respiration that was observed was not nearly as effective as the inhibition of ethylene formation observed in the same flowers (WU, ZACHARIAS, SALTVEIT and REID, 1992). This combination of prevention of ethylene formation and reduced respiration was thought to be responsible for the delay in senescence seen after ethanol application (HEINS and BLAKELY, 1980). It was proposed that since ethylene synthesis was associated with flower senescence (NICHOLS, 1968) and sucrose delayed it (MAYAK and KOFRANEK, 1976), the combination of the inhibition of ethylene, as well as a reduction in the respiration rate, was responsible for ethanol's senescence delaying effect. The ethanol may have acted as an energy source, or reduced the carbohydrate requirement of the flowers (HEINS and BLAKELY, 1980).

The aim of the research conducted in this Chapter was to determine other physiological responses of cut carnation flowers to ethanol treatment, as well as to attempt to clarify and corroborate what has been reported so far in the literature.

3.2. Materials and methods

3.2.1. Dry weight and moisture content

To determine any change in dry weight 3 flowers were harvested every 3 days, and the flower was divided into different parts (the major parts of a carnation flower are shown in FIGURE 3.1). The fresh weights of the petals, ovary and stem/calyx unit were recorded. These organs were then dried at 65°C for 24 h, and the dry weight was recorded. The moisture content of the tissue was determined by subtracting the DRY WEIGHT from the FRESH WEIGHT, and was expressed as a percentage (%).

3.2.2. Chlorophyll content

The chlorophyll a (Chl a), chlorophyll b (Chl b) and total chlorophyll content of ethanol-treated plant material was determined, according to the method of INSKEEP and BLOOM (1984, 1985), using the modifications introduced by ZOBOLO and VAN STADEN (1999). Flowers were harvested every 3 days, and their fresh weight recorded. The plant material was homogenised using a mortar and pestle, and 100 mg of the powder was extracted for 24 h in 10 mℓ N,N-dimethylformamide (DMF) for stems and calyxes (INSKEEP and BLOOM, 1985; ZOBOLO and VAN STADEN, 1999), while 50 mg ovary tissue was extracted in 3 mℓ DMF. The resultant homogenate was filtered through Whatman No. 1 filter paper and analysed for chlorophyll content. The absorbance of the extracts was measured in a Beckman DU-64 spectrophotometer at 647 nm (which is maximum

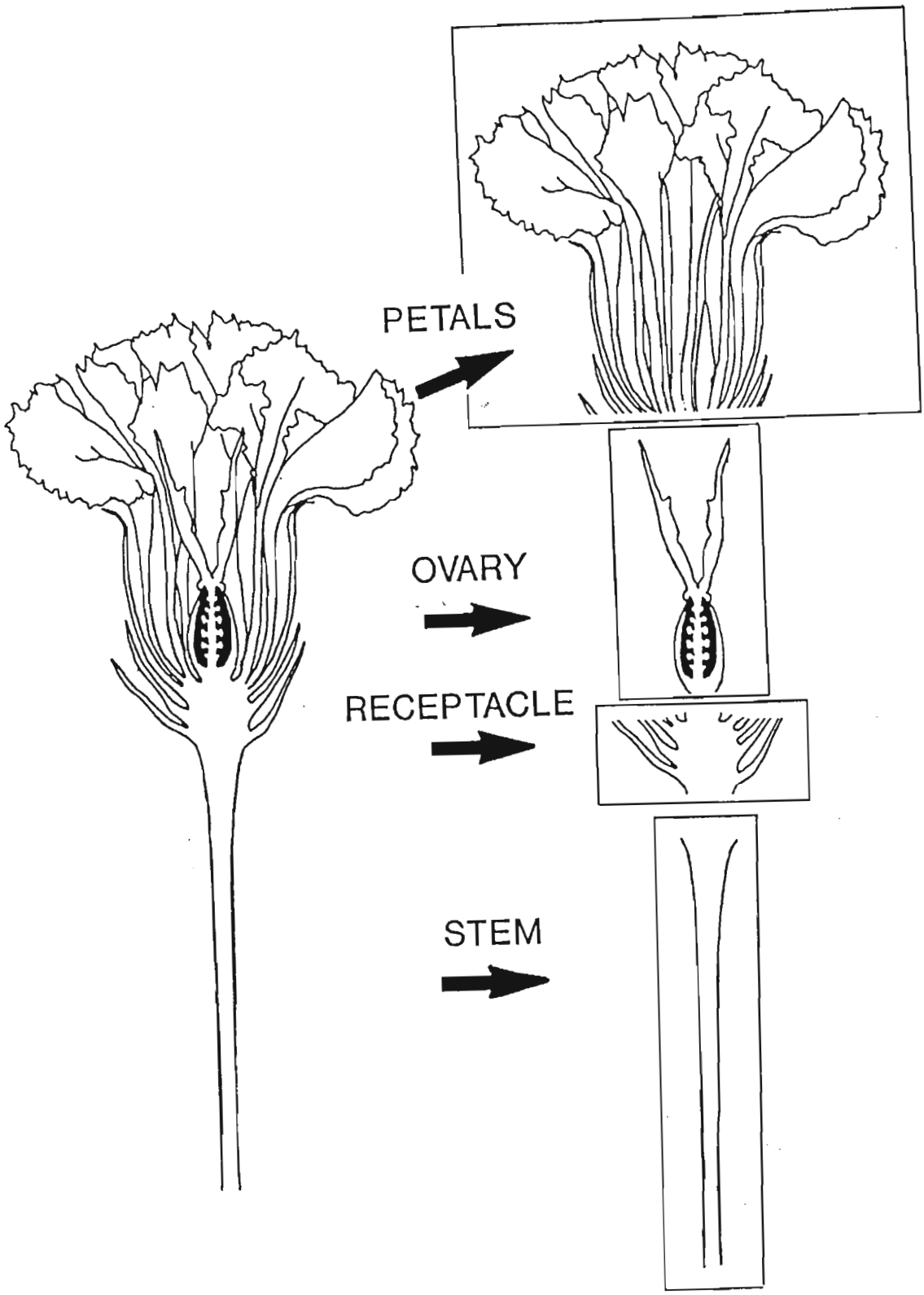


Figure 3.1. Diagram of a carnation (*Dianthus caryophyllus* L. cv. White Sim) flower showing the various plant parts that were harvested for data collection (After COOK 1984).

for chlorophyll b) and 664.5 nm (maximum for chlorophyll a) using a 1 mℓ quartz cuvette. Absolute chlorophyll concentrations (Chl a, Chl b and total chlorophyll) were quantified using the simultaneous equations of INSKEEP and BLOOM (1985). The experiment was repeated 3 times, and results were expressed as mg chlorophyll. plant part^{-1}.

$$\text{Chl A} = 12.70A_{664.5} - 2.79A_{647}$$

$$\text{Chl B} = 20.70A_{647} - 4.62A_{664.5}$$

$$\text{Total Chl} = 17.90\ A_{647} + 8.08\ A_{664.5}$$

3.2.3. Carbohydrate content

Ethanol was observed to inhibit the increase in ovary dry weight that was seen in normally senescing cut carnation flowers. This implied that the treatment in some way disrupted the source-sink dynamics. This made it necessary to determine the effect of ethanol treatment on the carbohydrate status of cut carnation flowers. The techniques used involved a silanisation step (SWEELEY, BENTLEY, MATIKA and WELLS, 1962) and an oxime synthesising step (TANOWITZ and SMITH, 1984). The method used was modified from the method of DREWES (1989). Flowers were treated with various concentrations of ethanol, as well as a water control. Three flowers from each treatment were harvested every three days, and divided into petals, ovaries and calyx/stem units. The fresh weight of these organs was recorded, after which material was flash frozen in liquid nitrogen. The frozen material was ground with a mortar and pestle, and 200 mg of the resultant powder

was extracted in 5mℓ 80% ethanol overnight on a shaker at 200 rpm. The resultant extract was centrifuged at 2 000g for 5 min., and 4 mℓ of the supernatant was dried down under nitrogen. Two hundred microlitres of a pyridine solution, containing 25 mg.mℓ^{-1} hydroxylamine monohydrochloride, was added and the resultant mixture was incubated at 37°C for 40 min. From this solution 100 µℓ was transferred to an Eppendorf tube and dried down under liquid nitrogen. The dehydrated solution was redissolved in 50 µℓ Sil-A and allowed to react for 15 min at room temperature. The samples were spun down in a millifuge for 10 s. One microlitre of the supernatant was subjected to gas chromatography using a Varian 3700 chromatograph fitted with a flame ionisation detector (using helium). The column was 2 m by 6 mm and packed with chromosorb warp mesh 80 – 100. The carrier gases were nitrogen and air. The injector port was set at 200°C and the detector at 300°C. The column temperature started at 125°C and ramped up to 270°C at a rate of 4°C.min^{-1}. The final temperature of 270°C was held for 10 min. A representative chromatogram is available in APPENDIX A.

Tentative identification of the sugars from the chromatograms was made possible using standard sugar samples at concentrations of 1 mg.mℓ^{-1}. The average retention time of a number of sugar standards was determined from results of 20 runs. These are listed in TABLE 3.1. The retention time of the peaks on the chromatograms produced from the flower samples could then be compared to these for tentative identification.

In order to determine if any deterioration had occurred within the samples during storage or preparation an internal standard was introduced. Phenyl-β-D-glycoside is a synthetic sugar that does not occur naturally in the tissues of cut carnation flowers. A number of chromatography runs were performed using 1μℓ of a 1mg.mℓ$^{-1}$ phenyl-β-D-glycoside solution, in order to determine a standard peak height, area and retention time (TABLE 3.1).

Table 3.1. The standard retention times of the 10 major sugar components of carnation flower tissues, and the internal standard Phenyl-β-D-glycoside.

Sugar	*Retention Time (min)*	*Standard Deviation*
Xylose	13.74	±0.03
Mannitol	19.32	±0.012
Sorbitol	19.61	±0.08
Fructose	20.367	±0.021
Sorbose	20.24	±0.14
Galactose	21.48	±0.05
Mannose	21.55	±0.05
Glucose	21.89	±0.26
Inositol	23.61	±0.21
Phenyl-β-D-glycoside	31.83	±0.12
Sucrose	36.17	±0.11

Once this was established, all plant samples were spiked with 1 mg.mℓ^{-1} phenyl-β-D-glycoside during the 80% ethanol extraction stage. Using the information gathered from the standard, it was possible to determine whether there was a reduction in the retention time or peak size of the sample, and this could be corrected for.

The silanisation step of the extraction technique is necessary in order to convert the oximes of the sugars to trimethyl derivatives, making them resistant to temperatures in excess of 250°C (SWEELEY, BENTLEY, MAKITA and WELLS, 1963). If not fully sialized the sugars break down under extreme temperatures, and two small peaks are detected by the integrator, instead of one large one (FIGURE 3.2). A problem with incomplete silanisation was noticed during the first repetition of this experiment. The amount of Sil A was doubled, to 100 μℓ, and this helped prevent double peak formation. A different sializing agent was then tested, Sylon BT. The chromatography peaks for the sugars treated with Sylon BT were found to be double the size of those from samples treated with Sil A (FIGURE 3.3). The first repetition of the experiment was completed using 100 μℓ Sil A. It was then repeated a further 3 times using 50 μℓ Sylon BT. A standard curve for sugar concentration was constructed using both Sil A and Sylon BT (FIGURE 3.4.). The relevant standard curve was used for the relevant sializing agent. The experiment was repeated four times. The figures were averaged, and the results expressed as μg sugar.g plant material^{-1}.

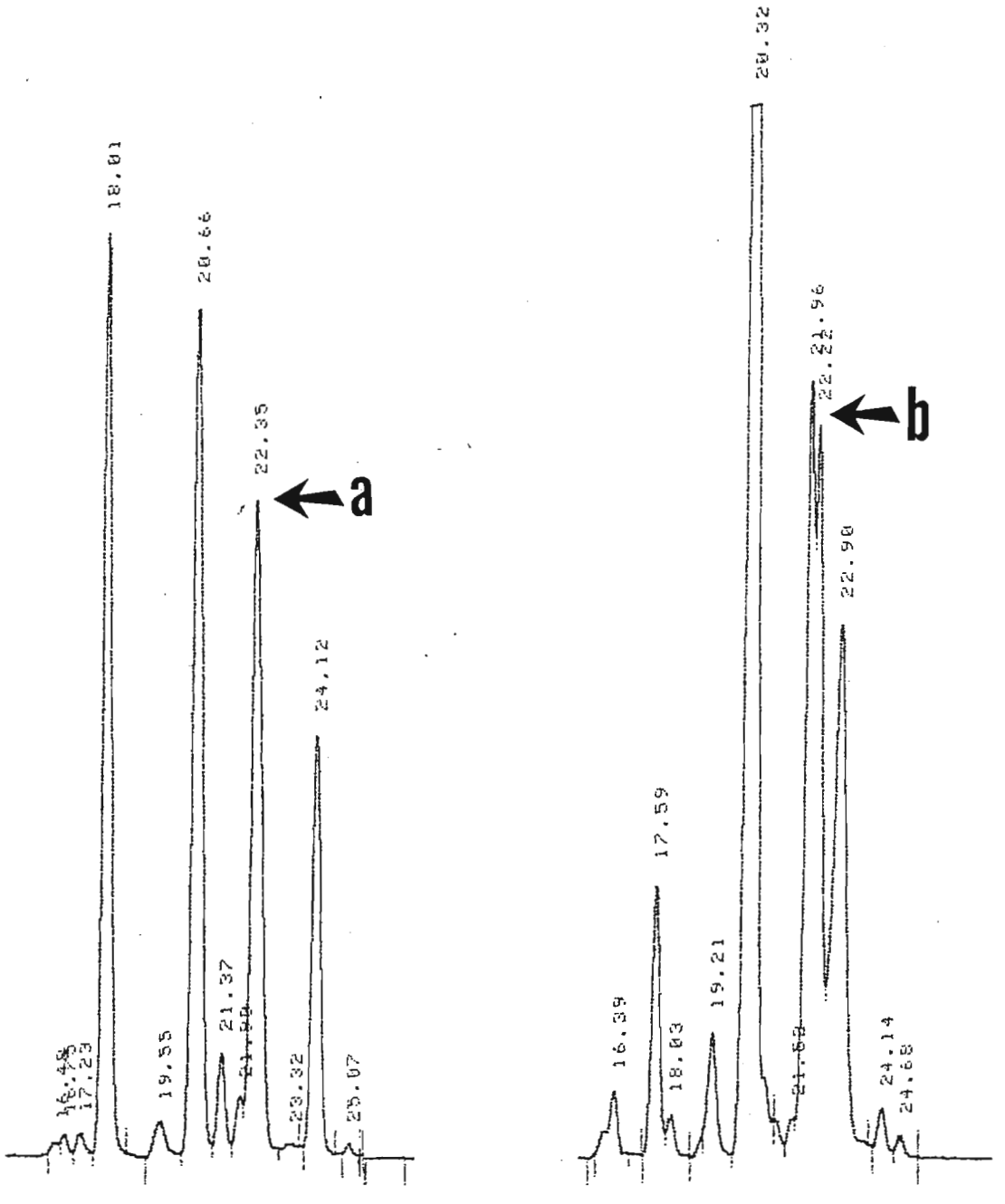


Figure 3.2. Chromatograms showing a fully sialized glucose peak (A) and an incompletely sialized glucose peak (B).

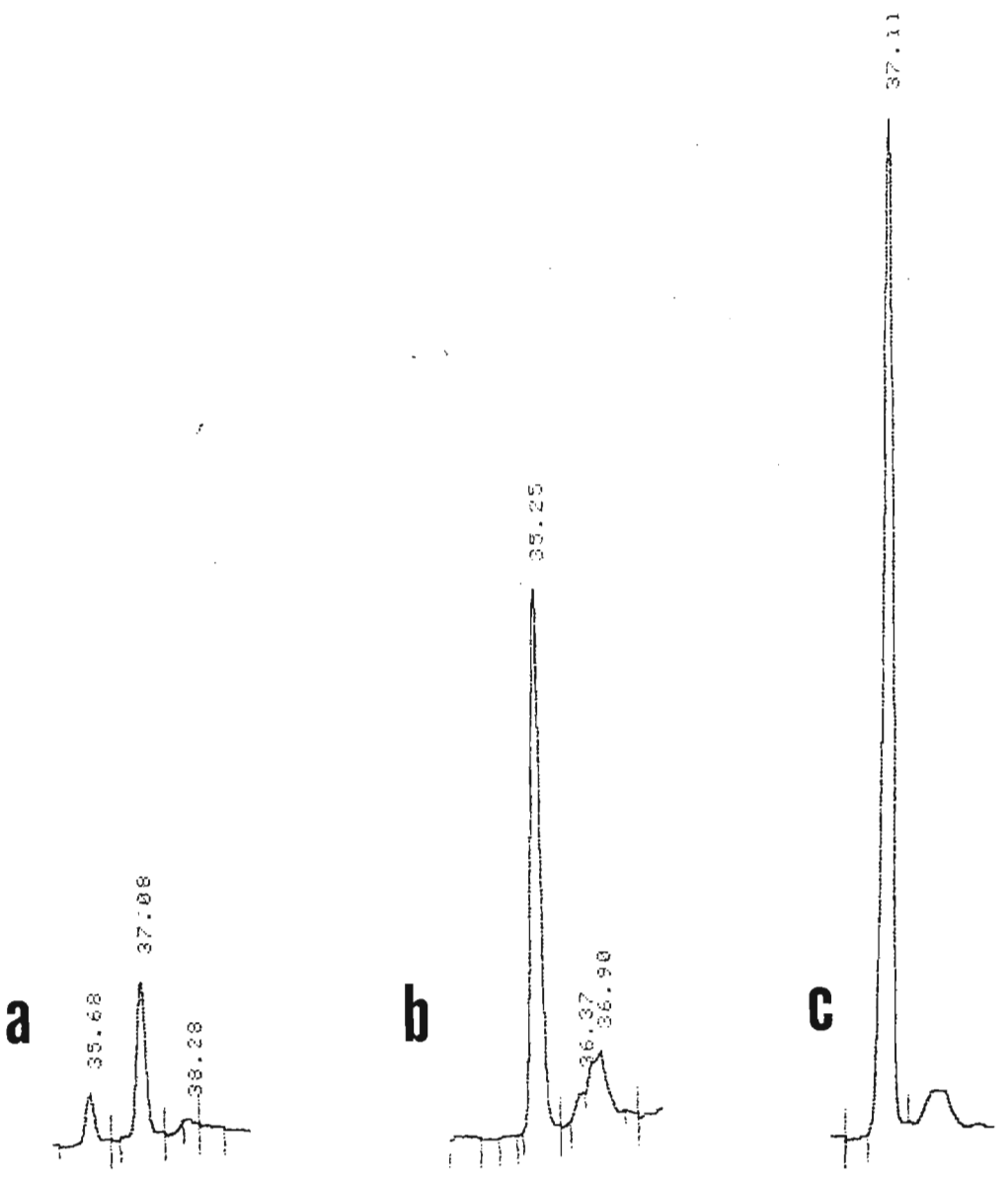


Figure 3.3. Chromatograms showing the same concentration of sucrose sialized with 50 µℓ Sil A (A), 100 µℓ Sil A (B) and 50 µℓ Sylon BT (C).

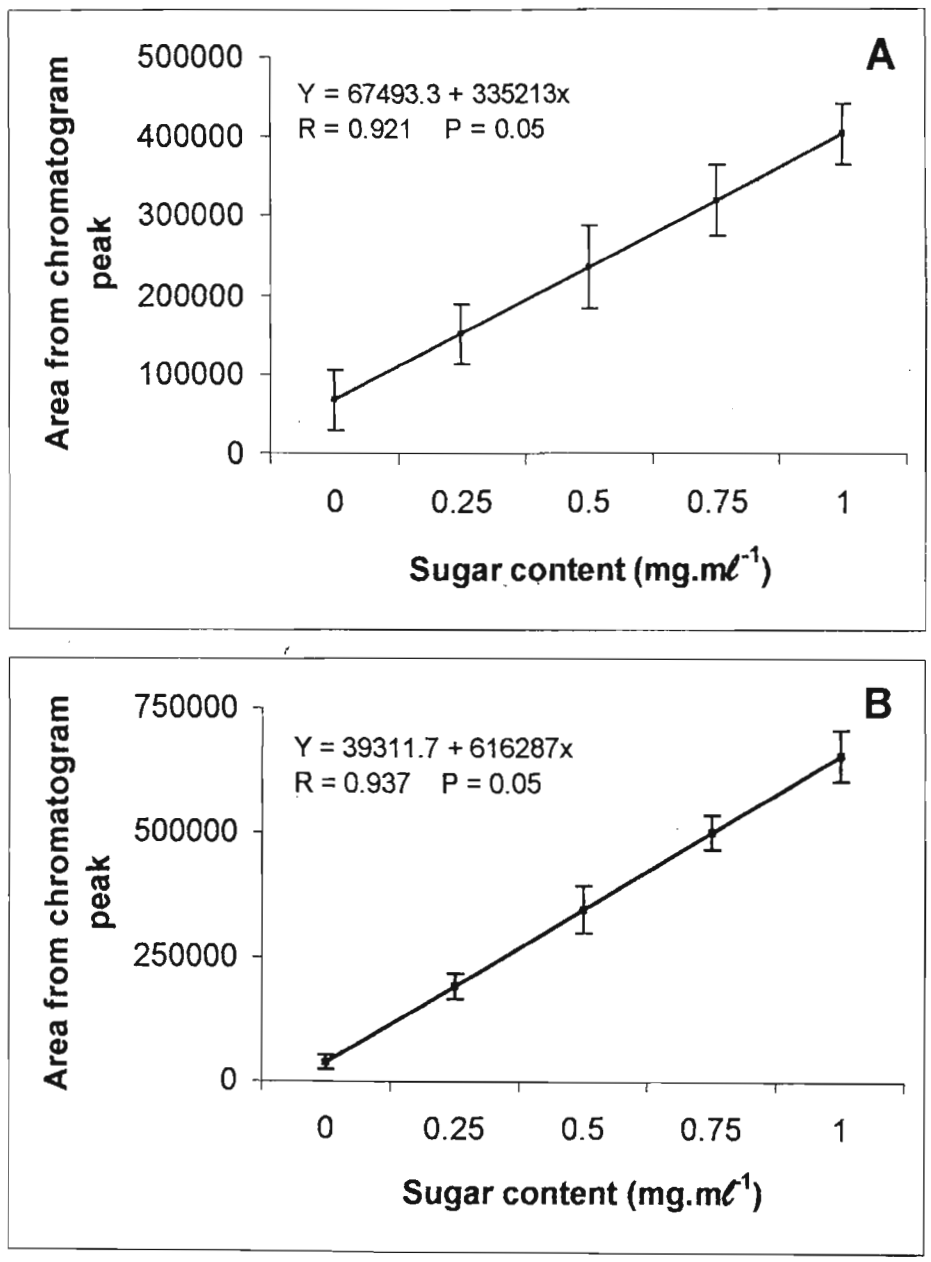


Figure 3.4. Standard curve for sugar content as detected by gas chromatography. A = sugars sialized with Sil A. B = sugars sialized with Sylon BT. Bars indicate standard errors.

3.2.4. The movement of ^{14}C sucrose

In order to determine the effect of ethanol on the sink activity of the ovary, flowers were treated with ubiquitously labelled ^{14}C sucrose, and its movement was traced. The sucrose, which had a specific activity of 552 $mCi.mM^{-1}$ was obtained from Amersham, UK. A spot was marked on one of the petals with marker pen, and this area was scraped with a syringe in order to damage the cuticle, and thereby facilitate sucrose uptake. A sucrose solution (10 $\mu\ell$) was applied to the marked petal with the syringe. This solution contained 100 000 dpm radiation, as well as a few drops of Tween 20 (a surfactant) to enhance sucrose uptake. The flowers were placed in either 3% ethanol or distilled water, and 3 flowers were harvested after 18 h and every second day thereafter. Harvested flowers were separated into petals, the marked petal, ovary and the stem/calyx unit. The fresh weight was recorded. The marked petal was dipped 3 times in a 10 $m\ell$ 10% ethanol solution to remove any unabsorbed ^{14}C, and this solution was kept separately. The plant material was dried for 48 h at 60°C, and the moisture content was determined. The dry material was flash frozen, ground using a mortar and pestle, and 10 mg of the resultant powder was added to 7 $m\ell$ scintillation vials. The material was digested for 3 h at 60°C using 0.5 $m\ell$ 1 : 1 H_2O_2 : perchloric acid (in the sealed scintillation vials). After cooling for 1 h, 4$m\ell$ Beckman Ready Solve EP® scintillation fluid was added to the vials, and radioactivity in the samples was determined using a Beckman LS 6000LL Scintillation Counter. At the same time 4$m\ell$ scintillation fluid was added to 0.5 $m\ell$ of the ethanolic solution that was used to wash the marked petal. This solution was combined with the marked petal extract,

and was counted in the scintillation counter as an indication of how much of the radioactivity was not taken up by the flower. After the initial counting, both sets of samples were left in the dark for 48 h and then counted again to rule out any background noise. Figures from the first and second counts were averaged, and the experiment was repeated 3 times. Results were calculated as % recovered radioactivity . plant part^{-1}.

3.2.5. The movement of ^{14}C ethanol

Ethanol-1-^{14}C was obtained from Sigma Chemical Company (St Louis, USA), with a purity of above 98% and a specific activity of 10.0 mCi.mM^{-1}. Each carnation flower was pulsed with 1 ml 3% ethanol, spiked with 50µCi ^{14}C ethanol. When the stems had taken up all of the pulse treatment, the flowers were placed in either an ethanol holding solution, or distilled water. Three flowers from each treatment were harvested every two days, and divided up into the inner and outer petals, ovary and stem/calyx unit. The fresh weight of these organs was recorded after which they were dried in a 65°C oven for 48 h and the dry weight recorded. Material was flash frozen with liquid nitrogen, and 250 mg of the ground material were extracted in 10 mℓ ethanol for 24 h. Three 1 mℓ samples of this ethanolic solution were combined with 4 mℓ Beckman Ready Solve EP® scintillation fluid in scintillation vials. The solution was subjected to radio-chemical analysis in a Beckman LS 6000 LL scintillation counter. The experiment was repeated three times. Combined results were calculated as the % recovered radioactivity.plant part^{-1}.

3.2.6. ACC content

Plant extracts were assayed for ACC using the technique of LIZADA and YANG (1979), whereby extracted ACC was converted to ethylene, which was detected by gas chromatography. Assays were performed in 12 x 7.5 mm test tubes, in which 1µM $HgCl_2$ and 500 µℓ plant extract were placed. The solution was brought to 900 µℓ with distilled water. The reaction vessels were sealed with rubber stoppers and kept on ice. Approximately 100 µℓ of a cold mixture of 5% NaOCl and saturated NaOH (2:1, v/v) which contained 45 µM NaOCl, was injected through the rubber stopper with a 100µℓ syringe. Tubes were then vortexed for 5 sec, kept on ice for 2.5 min, and then vortexed again. A syringe was inserted through the stopper, and a 1 mℓ sample was removed from each tube, and subjected to gas chromatography, as described in Chapter 2.2. (LIZADA and YANG, 1979).

A number of techniques for the extraction of ACC have been published in the last 20 years. The older ones involve long term extraction techniques, using either 5% sulfosalicylic acid (LIZADA and YANG, 1979; YU, ADAMS and YANG, 1979), 3% perchloric acid (BUFLER and MOR, 1980; MOR, HALEVY, SPIEGELSTEIN and MAYAK, 1985), or 80% ethanol (BUFLER and MOR, 1980; BUFLER, MOR, REID and YANG, 1980; McKEON, HOFFMAN and YANG, 1982; NIEDER, YIP and YANG, 1986). Ethanol extractions replaced both sulfosalicylic acid and perchloric acid as the most popular ACC extraction technique. It was determined that the presence of ethanol in the assay mixture resulted in ethylene being produced from a source other than ACC (BUFLER and MOR, 1980). In the presence of nitrates or amines, but the

absence of ACC, ethanolic extracts produced ACC when assayed (NIEDER, YIP and YANG, 1986). If the sample was evaporated to remove all traces of ethanol, sensitivity to the assay was increased (BUFLER and MOR, 1980). Much shorter ACC extraction techniques are now being used, including trichloroacetic acid (TCA) (SINGH, ELENSEN and KAO, 1992) and sodium phosphate ($NaPO_4$) buffer (KNEISSEL and DEIKMAN, 1996). Neither of these techniques have however, been used to extract ACC from cut carnation flower material.

It was decided to test these five extraction techniques on ethanol-treated cut carnation flowers in order to determine which gave the best results. Flowers were treated with 3% ethanol solutions, and harvested every two days. The fresh and dry weight of the plant parts was recorded, before the plant material was flash frozen with liquid nitrogen and ground using a mortar and pestle. Powdered plant material (250 mg) was extracted using each of the extraction techniques.

Extraction with 5% sulfosalicylic acid

Plant material was extracted in 500 mℓ for 24 h at 4°C, as per the technique of LIZADA and YANG (1979), with some minor alterations. The plant material was then centrifuged for 30 min at 15 000g in a Beckman Avanti j-25 I centrifuge with a JA 14 rotor. The supernatant was then passed through a column (1 x 6 cm) of ion exchange resin (Dowex 50, H^+) which was washed with water. Amino acids were eluted from the column with 20 mℓ 2 N NH_4OH. After concentration *in vacuo* at 38°C eluates were assayed for ACC (LIZADA and YANG, 1979).

Extraction with 3% perchloric acid

After grinding, the plant material was extracted in 1 mℓ 3% perchloric acid for 48 h at 4°C (MOR, HALEVY, SPIEGELSTEIN and MAYAK, 1985). The material was centrifuged at 25 000g for 10 min, and the supernatant was assayed for ACC.

Extraction with 80% ethanol

Plant material was extracted overnight in 750 mℓ 80% ethanol, and the resultant extract was centrifuged at 25 000g for 15 min. The pellet was redissolved in another 750 mℓ ethanol, and extracted for a further 18 h, before being re-centrifuged. The supernatants were combined and evaporated to dryness *in vacuo*. The dry residue was redissolved in water, and this aqueous solution was assayed for ACC (BUFLER and MOR, 1980).

Extraction with TCA buffer

Material was extracted in 2 mℓ 0.2 M trichloroacetic acid (TCA). After 2 h at 4°C the extract was centrifuged at 15 000g for 15 min to remove cellular debris. The supernatant was then assayed for ACC (SINGH, EVENSEN and KAO, 1992).

Extraction with Na_2PO_4 buffer

The powdered plant material was extracted on ice for 2 h in 1 mℓ 0.2 mM sodium phosphate (Na_2PO_4) buffer (pH 8.0). The extract was centrifuged at 15 000g for 30 min and assayed directly for ACC (KNEISSEL and DEIKMAN, 1996). To prepare a 0.2 M Na_2PO_4 buffer with a pH of 8.0 at 25°C, it is necessary to add 186.4

mℓ 1 M Na_2HPO_4 to 13.6 mℓ 1 M NaH_2PO_4 and make up the solution to 1ℓ (SAMBROOK, FRITSCH and MANIATIS, 1989).

The five extraction techniques were performed on the same plant material, and the results were expressed as µℓ ethylene recorded.g plant material^{-1}, and are shown in FIGURE 3.5. The Na_2PO_4 buffer yielded the best results, and was subsequently used for the routine extraction of ACC from ethanol-treated cut carnation flowers. A standard curve for ACC concentration was established (FIGURE 3.6.) using Na_2PO_4 buffer as background. The experiment was repeated 3 times, and the results averaged. Results were expressed as µM.g plant material^{-1}.

3.2.7. ACC oxidase activity

The activity of ACC oxidase is calculated using the extracts that were used to determine ACC content. Once the amount of ACC in the tissue has been determined, the efficiency of the conversion of ACC to ethylene can be measured. Internal standards of ACC are used, and the percentage recovery of the internal standard combined with the internal ACC level is calculated (LIZADA and YANG, 1979). Five internal standards were used for these experiments (10, 20, 30, 40 and 50 µM ACC). Results are expressed as percentage activity.

3.2.8. Protein extraction and quantification

The protein content of ethanol-treated flowers was determined using the Bio-Rad protein assay, also known as the Bradford protein assay. This is a dye-binding assay based on the differential colour change of a dye in response to various

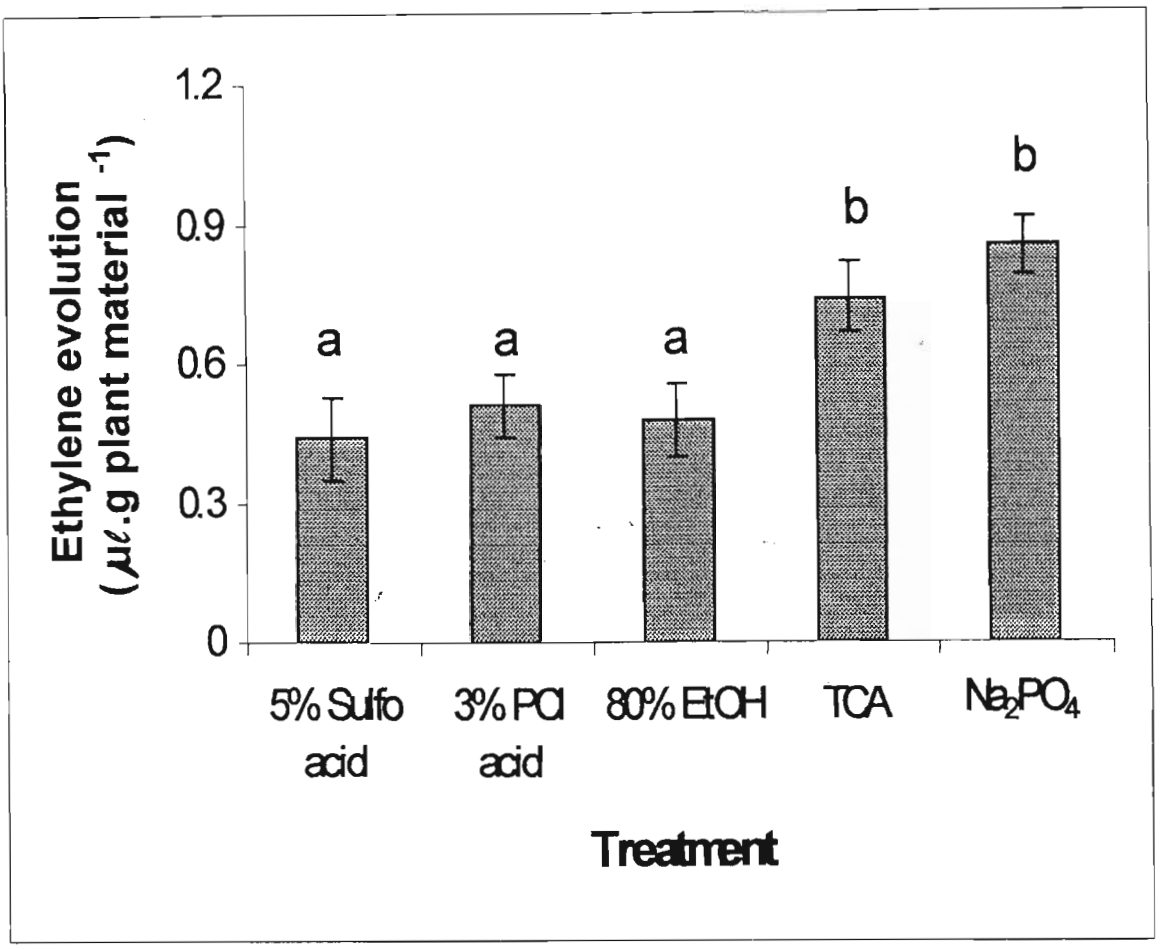


Figure 3.5. ACC levels from the same plant material extracted using the various ACC extraction techniques. 5% Sulpho acid = 5% Sulfosalicylic acid; 3% PCl acid = perchloric acid; 80% EtOH = 80% Ethanol; TCA = 0.2 M trichloroacetic acid and Na_2PO_4 = in 1 mℓ 0.2 mM sodium phosphate buffer. Bars indicate standard errors. Bars with the same letters are not significantly different from one another.

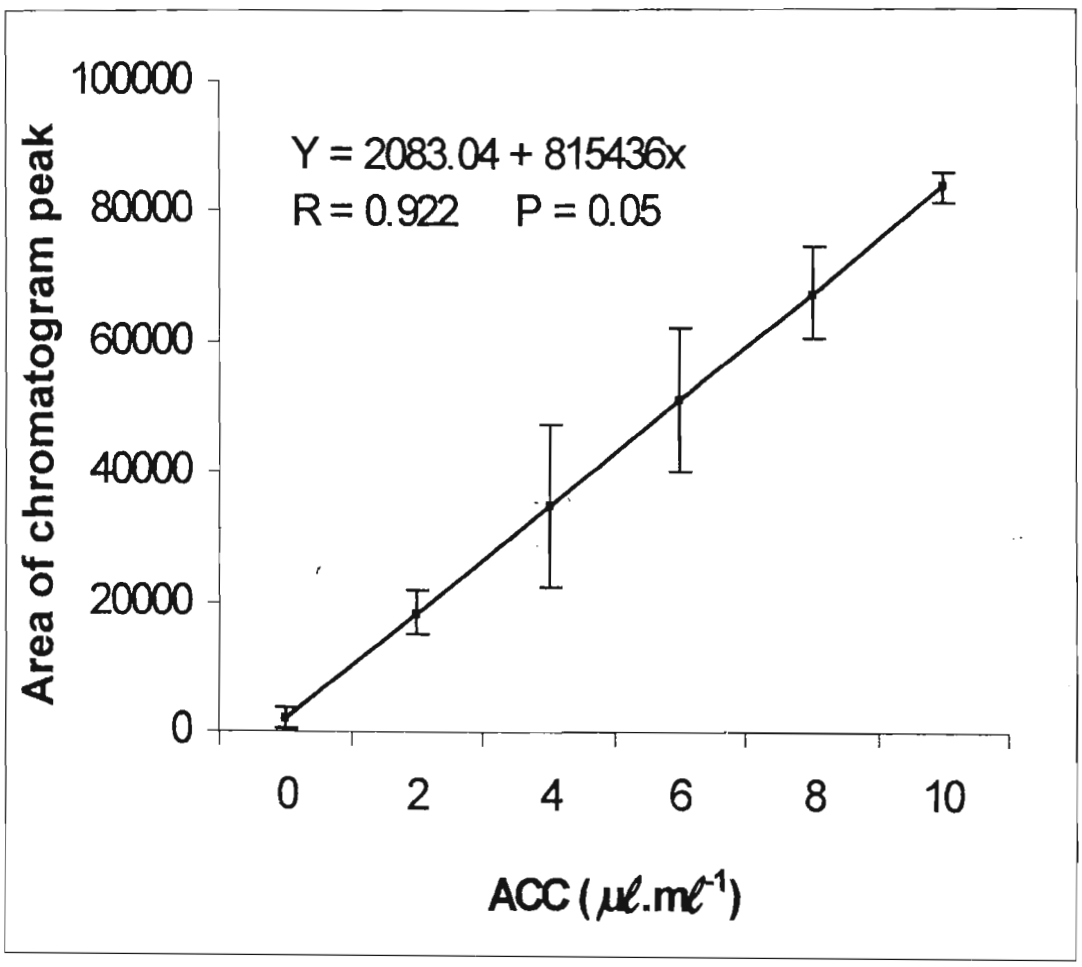


Figure 3.6. ACC standard curve, as detected by gas chromatography. Bars indicate standard errors.

concentrations of protein (BRADFORD, 1976). The assay is based on the principle that the maximum absorbance of Coomassie Brilliant Blue moves from 465 nm to 595 nm when bound to a protein. It is quick and easy to perform, requiring only 5 min and one reagent. Plant extract (20 µℓ) were placed in a plastic cuvette. Added to this was the pre-made dye reagent, which contains coomassie, phosphoric acid and methanol. The dye reagent is provided at 5-fold concentrate, so it is necessary to dilute 1mℓ concentrate with 4 mℓ distilled water. The solution required filtering through Whatman No. 1 paper to ensure it was free of contaminants. The dilute reagent (1mℓ) was added to the plant extracts. The entrance of the cuvette was covered with parafilm in order to mix the solution. The sample was then left to stand for 5 min, before being analysed using a Varian Cary 50 conc UV – visible spectrophotometer, fitted with Cary Win UV software, at 595 nm. A protein standard curve was constructed using bovine-serum A (BSA) as a standard (FIGURE 3.7.). Results were expressed as µg protein.plant part^{-1}.

In order to obtain extracts, the plant material was flash frozen with liquid nitrogen, and ground with a mortar and pestle. The resultant powder (250 mg) was extracted in 1 mℓ phosphate buffer solution, containing 5M NaCl, 0.1 M dithiothreitol (DTT), 0.5 M ethylene diamine tetraacetic acid (EDTA), 100 mM phenylmethylsulfonyl fluoride (PMSF) and 1.5% polyvinylpolypyrollidone (PVPP) for 30 min (on ice). The extract was centrifuged at 10 000 g for 10 min, where after the supernatant was removed and spun down at 1 500g in a Sigma 113 desktop microfuge for 10 min (modified from HILLS, 1999). The extract (20µℓ) was then

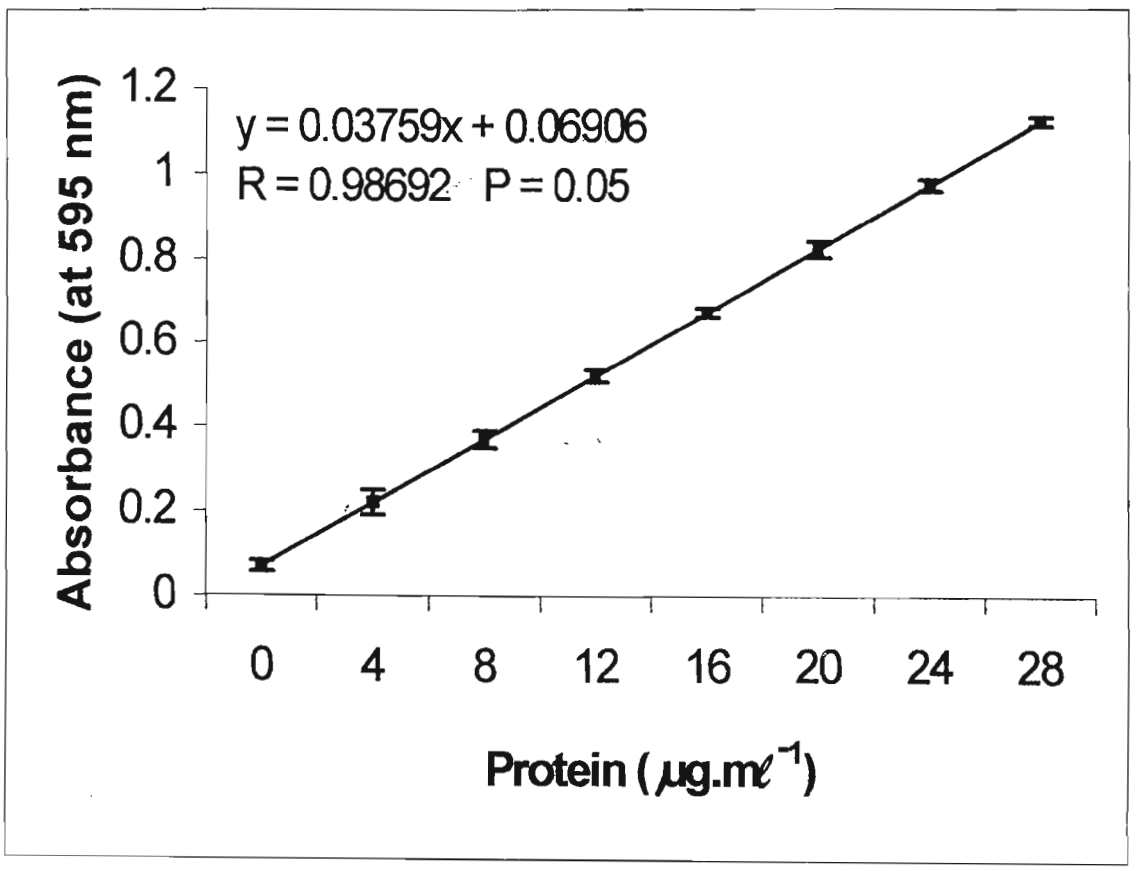


Figure 3.7. Standard protein curve, as detected by spectrophotometry. Bars indicate standard errors.

assayed as described previously.

3.2.9. Polyacrylamide gel electrophoresis of proteins

The extracted and quantified proteins were separated out using gel electrophoresis, in order to determine whether changes in protein were general changes, or changes in a specific size and type of protein. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to SMITH (1994), a method based on the original LAEMMLI (1970) protocol. Proteins were separated on a 1.5 mm thick gel consisting of a stacking gel (pH 6.8) and a separating gel (pH 8.8). A stock acrylamide mixture (total acrylamide content % T = 30%, ratio of cross-linking agent to acrylamide monomer, % C = 2.7%) was prepared by dissolving 73 g acrylamide and 2 g N'N' methylenebisacrylamide (*bis*-acrylamide) in 250 mℓ deionised distilled water (dd$H_2$0). The stock was filtered through Whatman No. 1 filter paper to remove insoluble impurities. It was stored in the dark at 4°C and remained stable for one month. Separating gel buffer stock was prepared from 1 g SDS and 45.5 g 2-amino-2(hydroxymethyl)-propane-1,3-diol (Tris) dissolved in 250 mℓ ddH20, with pH adjusted to 8.8 with HCl. Stacking gel buffer stock was prepared by dissolving 1 g SDS and 15.1 g Tris in 250 mℓ ddH20, with a pH of 6.8. Both buffer stocks were stored at 4°C, being stable for several months at that temperature. The pH of the stacking gel buffer was checked each time before use, and adjusted with HCl if necessary (HILLS, 1999).

Separating gel solution (30 mℓ) was prepared from 15 mℓ acrylamide stock, 7.5 mℓ ddH2O and 7.5 mℓ separating gel buffer. This solution was degassed under vacuum and polymerisation initiated by the addition of 45 µℓ 10% ammonium persulfate (APS) and 15 µℓ NNN'N'tetramethylethylene-diamine (Temed). The gel was poured between two ultra-clean glass plates, prepared by washing each one twice with warm, soapy water, followed by two washes with absolute alcohol and one wash with acetone. Plates were buffed using clean, lint-free towels. The gel was allowed to polymerise for 1 h after the addition of a ddH_20 overlay. This was poured off after the gel had completely polymerised. The stacking gel solution was prepared by mixing 750 µℓ acrylamide stock and 3 mℓ ddH20. After degassing, 1.25 mℓ stacking gel buffer was carefully added and polymerisation was initiated by adding 15 µℓ 10% [w/v] APS and 5 µℓ Temed. The stacking gel was poured on top of the separating gel, and a comb inserted between the glass plates to form the loading wells. The stacking gel extended below the comb to a depth of 1 cm (HILLS, 1999).

Protein extracts stored at –20°C were thawed, and aliquots containing 20 µg protein were made up to 60 µℓ with ddH_20. These samples were then mixed with 20 µℓ of double strength sample solvent (4.6% [w/v] SDS, 10% [v/v] 2-mercaptoethanol, 0.125 M Tris-Cl (pH 6.8), 0.01% [w/v] bromophenol blue, 20% [v/v] glycerol). A mixture of proteins of known weight (in daltons Da) from the Combithek Calibration Protein kit (Boehringer-Mannheim) was also prepared and electrophoresed in the outer lanes of each gel to allow for size determination of unknown proteins (TABLE 3.2) (HILLS, 1999). Samples were electrophoresed at a constant current of 10

milliamps (mA) through the stacking gel. Once the dye front reached the interphase between the stacking gel and the separating gel, the current was increased to 25 mA until the bromophenol blue reached the end of the gel (HILLS. 1999).

For the presentation of results the gels were cut to display only the relevant lanes. The original gels are available in Appendix B.

3.2.10. Detection of electrophoresed proteins

Proteins separated on gels were detected by silver-staining. The gels were subjected to a two-phase pre-fixing step consisting of a 30 min wash in a solution containing 30% (v/v) ethanol and 10% (v/v) glacial acetic acid, followed by a second 30 min wash in 30% ethanol - 7% acetic acid solution. Gels were fixed for 30 min in a 10% (v/v) glutaraldehyde solution. After fixing, the gels were soaked overnight in ddH_20. Before staining the gels were rinsed for a further 30 min in fresh ddH_2O, as well as being washed with 5 $\mu g.m\ell^{-1}$ DTT. Gels were stained for 30 min in a 0.5% (w/v) solution of silver nitrate and then rinsed well with water to remove any unbound silver. To develop the stain, the gels were soaked in developing solution (3% [w/v] sodium carbonate, 0.0005% [v/v] formaldehyde) until the desired level of development was achieved. The reaction was then terminated by adding 10 $m\ell$ acetic acid and agitating for 10 min. Finally, gels were rinsed with several volumes of ddH_20. After being photographed the stained gels were stored in a small amount of ddH_20 in a sealed plastic bag at 4°C (HILLS, 1999).

Table 3.2. Details of proteins from the Combithek Calibration Proteins Kit (Boeringer-Mannheim) and volumes used in preparation of the molecular weight marker mixture for polyacrylamide gel electrophoresis (After Hills, 1999).

Protein	*Concentration* ($mg.m\ell^{-1}$)	M_r (*Da*)	*Volume* ($\mu\ell$)
β-Galactosidase	0.5	116 400	0.5
Fructose-6-phosphohate kinase	0.65	85 200	0.39
Glutamatecdehydrogenase	0.5	55 600	0.5
Triose-phosphate isomérase	0.5	26 600	0.25
Aldolase	0.65	39 200	0.39
Trypsin inhibitor	0.8	20 100	0.16
Lysozyme	0.5	14 300	0.25

3.3. Results

3.3.1. The effect of ethanol treatment on the physical appearance of cut carnation flowers

Cut carnation flowers exhibit specific physical changes during senescence. As the flower senesces, so the petals roll inwards, and become limp. The ovary gains weight. increases in diameter and becomes greener. However, when the vase life of cut carnation flowers was increased by ethanol treatment, no petal in-rolling was observed. Instead the petals appeared burnt (PLATE 3.1). The enlarging and greening of the ovary was also inhibited (PLATE 3.2). This implies that ethanol treatment is affecting the weight of both the petals and the ovary. Variations in dry weight indicate changes in moisture and solid content of organs, and are evident in the changes in size and shape. An investigation into the effect of ethanol treatment on the dry weight of cut carnation flowers was performed. It was necessary to record the longevity of the flowers used in this experiment in order to correlate any changes in weight with the progression of senescence. Longevity is presented in TABLE 3.3.

Table 3.3. Longevity of flowers (used for dry weight and moisture content experiments) treated with various concentrations of ethanol.

	Ethanol concentration (%)						
	0	***1***	***3***	***5***	***8***	***10***	***20***
S50 (days)	7	13	15	11	13	12	10

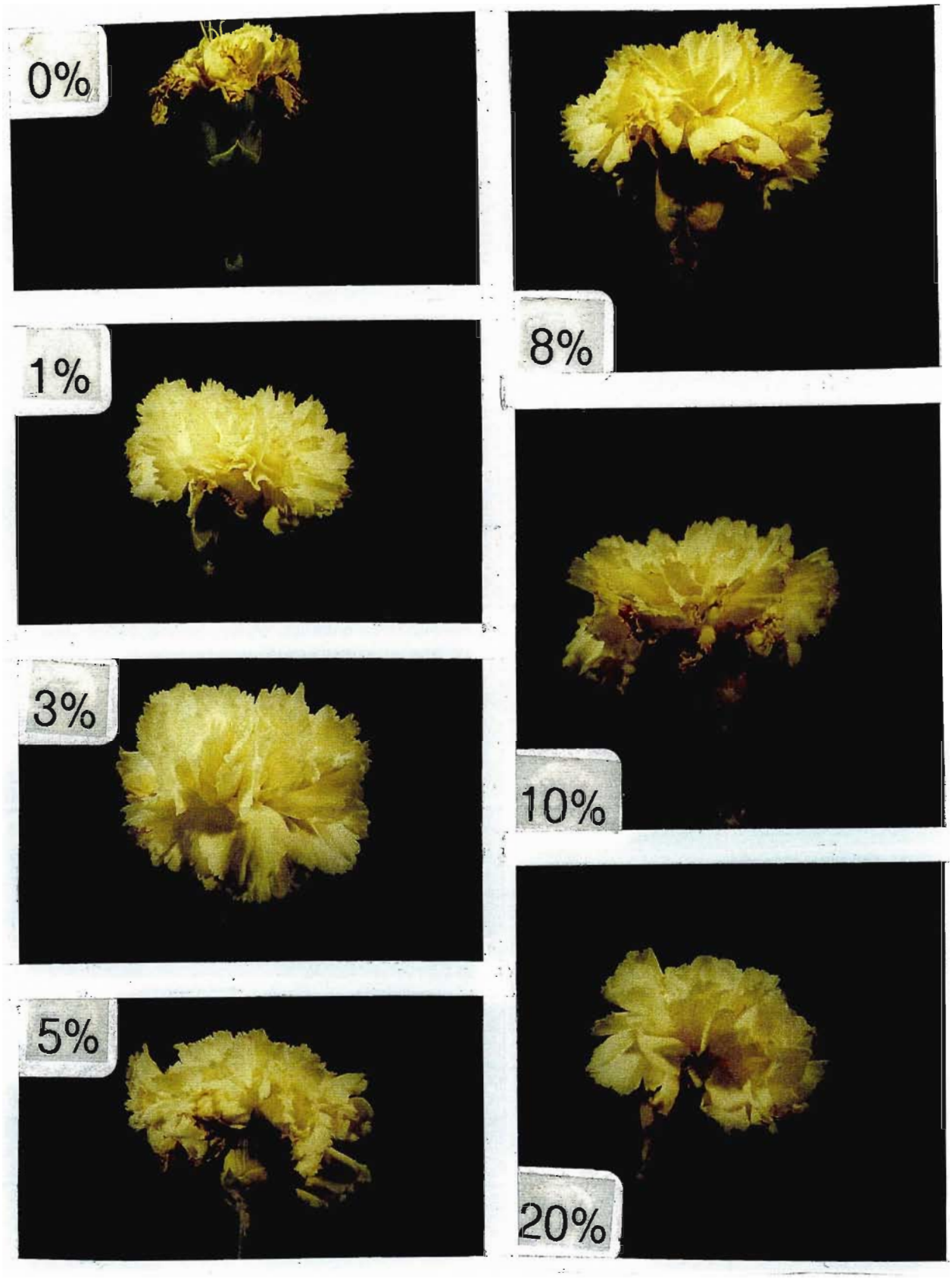


Plate 3.1. The appearance of ethanol-treated cut carnation flowers ten days after harvest. Test boxes indicate ethanol concentration. Note the petal in-rolling in the control and the increase in the degree of petal burning as the ethanol concentration increases.

Plate 3.2. The appearance of ovaries of cut carnation flowers ten days after harvest. Test boxes indicate the ethanol concentration. Note the change in ovary size and colour as ethanol concentration increases.

The effect of ethanol treatment on the dry weight (TABLE 3.4.) and moisture content (TABLE 3.5.) was determined.

Table 3.4. The effect of ethanol on the dry weight (g) of petals of cut carnation flowers. Standard errors are indicated in brackets. Results with the same letters are not significantly different from one another.

Time after the onset of treatment (days)	Ethanol concentration (%)						
	0	1	3	5	8	10	20
0	1.042 (0.087a)	1.042 (0.087a)	1.042 (0.087a)	1.042 (0.087a)	1.042 (0.087a)	1.042 (0.087a)	1.042 (0.087a)
3	1.023 (0.87a)	1.021 (0.91a)	1.013 (1.04a)	1.002 (0.87a)	1.004 (0.85a)	1.047 (0.97a)	1.014 (0.73a)
6	0.8896 (0.076a)	1.044 (0.088b)	1.0482 (0.122b)	0.9305 (0.11ab)	1.042 (0.11ab)	0.975 (0.13ab)	0.952 (0.107a)
9	0.7877 (0.124a)	0.729 (0.2a)	0.999 (0.085b)	0.973 (0.1ab)	1.0108 (0.15ab)	1.0158 (0.069b)	0.925 (0.15ab)
12	0.759 (0.054a)	0.914 (0.09 b)	0.8699 (0.15 b)	0.935 (0.064b)	0.9555 (0.131b)	0.9363 (0.082b)	0.9256 (0.072b)
15	0.727 (0.038a)	0.9556 (0.111b)	0.9122 (0.092b)	0.914 (0.124b)	0.927 (0.125b)	0.922 (0.088b)	0.929 (0.067b)

Given the visual change in the petals of cut carnation flowers during senescence compared to control flowers, it was necessary to investigate the effect of ethanol treatment on the dry weight of the petals. The dry weight of the control flowers decreased steadily after initial harvesting, and by the time S50 occurred, the dry weight of the control petals was only 75% of the original weight. This indicated large-scale movement of cellular contents.

Table 3.5. The effect of various concentrations of ethanol on the moisture content (%) of petals of cut carnation flowers.

Time after the onset of treatment (days)	Ethanol Concentration (%)						
	0%	1%	3%	5%	8%	10%	20%
0	84.9	84.9	84.9	84.9	84.9	84.9	84.9
3	85.0	84.6	85.7	85.4	84.2	84.8	84.5
6	85.7	84.9	86.1	86.2	85.2	84.9	84.5
9	69.2	85.0	86.6	82.5	80.8	81.2	81.3
12	16.9	83.6	83.8	74.3	81.7	81.2	81.4
15	19.0	80.5	79.7	74.0	81.0	79.7	80.0

These petals also showed huge decreases in moisture content, particularly by the 12th day of the experiment when the flowers were in the advanced stages of senescence. In treated flowers however, there was no major change in the dry weight of the petals after harvesting. Even on the 15th day of the experiment, the greatest loss in petal dry weight was only 12% of the original weight (for 5% ethanol). The concentration of ethanol used appeared to have no significant effect on the prevention of dry weight loss. In ethanol-treated flowers there was almost no change at all in the moisture content of the petals, even after S50 had been reached, the petals remained moist.

The effect of various concentrations of ethanol on the dry weight (TABLE 3.6) and moisture content (TABLE 3.7) of the ovary was recorded.

Table 3.6. The effect of ethanol on the dry weight (g) of ovaries of cut carnation flowers. Standard errors are indicated in brackets. Results with the same letters are not significantly different from one another.

Time after the onset of treatment (days)	Ethanol concentration (%)						
	0	1	3	5	8	10	20
0	0.087 (0.184a)	0.087 (0.184a)	0.087 (0.184a)	0.087 (0.184a)	0.087 (0.184a)	0.087 (0.184a)	0.087 (0.184a)
3	0.1211 (0.012a)	0.088 (0.02b)	0.087 (0.015b)	0.083 (0.014b)	0.081 (0.013b)	0.068 (0.11b)	0.064 (0.094b)
6	0.1292 (0.032a)	0.086 (0.018b)	0.085 (0.006b)	0.082 (0.019b)	0.0727 (0.01bc)	0.0625 (0.013c)	0.055 (0.013c)
9	0.1317 (0.021a)	0.0709 (0.015b)	0.0552 (0.018b)	0.0694 (0.016b)	0.0706 (0.021b)	0.061 (0.011b)	0.056 (0.015b)
12	0.113 (0.029a)	0.057 (0.01b)	0.064 (0.024b)	0.089 (0.025b)	0.058 (0.02b)	0.057 (0.012b)	0.059 (0.017b)
15	0.0935 (0.025a)	0.077 (0.042b)	0.053 (0.011b)	0.059 (0.02b)	0.054 (0.012b)	0.061 (0.03b)	0.058 (0.021b)

The ovaries of control flowers gain weight and diameter during senescence (NICHOLS and HO, 1975a; COOK and VAN STADEN, 1986; VAN STADEN, 1995). During experimentation the ovaries of the control flowers increased in dry weight, and on the 9th day after harvest were 150% heavier than when the experiment began. Towards the end of the experiment the weight of control ovaries did begin to decrease. This decrease was most likely due to cellular breakdown, as at this point the control flowers had been dead for several days. In the ethanol-treated flowers no increase in ovary dry weight occurred. In fact large losses were observed, including 40% of the ovary dry weight of the flowers treated with 3% ethanol having been lost

on day 15. Ovary dry weight decreased significantly compared to the water control for all concentrations of ethanol applied. No significant variation was observed within the ethanol concentrations applied. These results not only show that ethanol disrupted the sink activity of the ovaries of ethanol-treated flowers, but that some other physiological disturbance also occurred that resulted in a decrease in ovary dry weight.

Table 3.7. The effect of various concentrations of ethanol on the moisture content (%) of ovaries of cut carnation flowers.

Time after the onset of treatment (days)	Ethanol concentration (%)						
	0%	1%	3%	5%	8%	10%	20%
0	85.4	85.4	85.4	85.4	85.4	85.4	85.4
3	84.2	85.5	86.9	85.6	85.9	83.7	83.4
6	84.0	86.0	87.7	85.3	85.7	82.6	80.2
9	85.3	86.8	89.6	86.6	85.0	83.0	78.0
12	85.1	84.0	86.2	84.6	85.4	84.7	77.4
15	85.9	78.4	82.8	92.2	84.4	80.5	71.8

As for the moisture content of the ovaries, little change was seen in control or treated-flowers. This means that, as expected, the increases in ovary size and diameter were due to changes in the dry matter content i.e. the importation of carbohydrates from other organs of the flower. The damaged appearance and reduction in size of treated ovaries was therefore also due to changes in the dry matter content of the ovaries. There was a decrease in the dry matter content of the

petals and the ovary of ethanol-treated flowers, once again indicating the disruption of the source-sink activity within senescing cut carnation flowers

Another effect of ethanol treatment on the physical appearance of cut carnation flowers was a change in the colour of the ovaries (PLATE 3.2) calyx and stem, particularly at the nodes (PLATE 3.3). These became more yellow/brown than green, suggesting a change in the chlorophyll content of the tissues. The total chlorophyll content of these organs was determined, and is presented as FIGURE 3.8. Despite a visual change in the colour of the stems, and chlorophyll levels being slightly lower than the control, no significant reduction in the chlorophyll content of the stems treated with 1% and 3% ethanol was recorded. At a concentration of 8%, ethanol did result in a substantial decrease in chlorophyll compared to the control flowers throughout the experimental period. In general, the chlorophyll content of the stems of cut carnation flowers decreased during senescence, regardless of whether they had been treated with ethanol or not.

In the calyx and ovary, however, an increase in the total chlorophyll content was seen in control flowers. In the calyx, the chlorophyll content increased until day 6, after which it decreased. At this point the flowers had reached advanced senescence, and the loss of chlorophyll coincided with the degeneration of the chloroplasts and loss of membrane integrity (BUTLER and SIMON, 1970; THIMANN, 1980; WOOLHOUSE 1984; GEPSTEIN, 1988). In the ethanol-treated flowers, regardless of concentration, there was a significant decrease in the chlorophyll content of the calyx compared to the water control. The same can be said of the chlorophyll content of the ovary. In control ovaries the chlorophyll

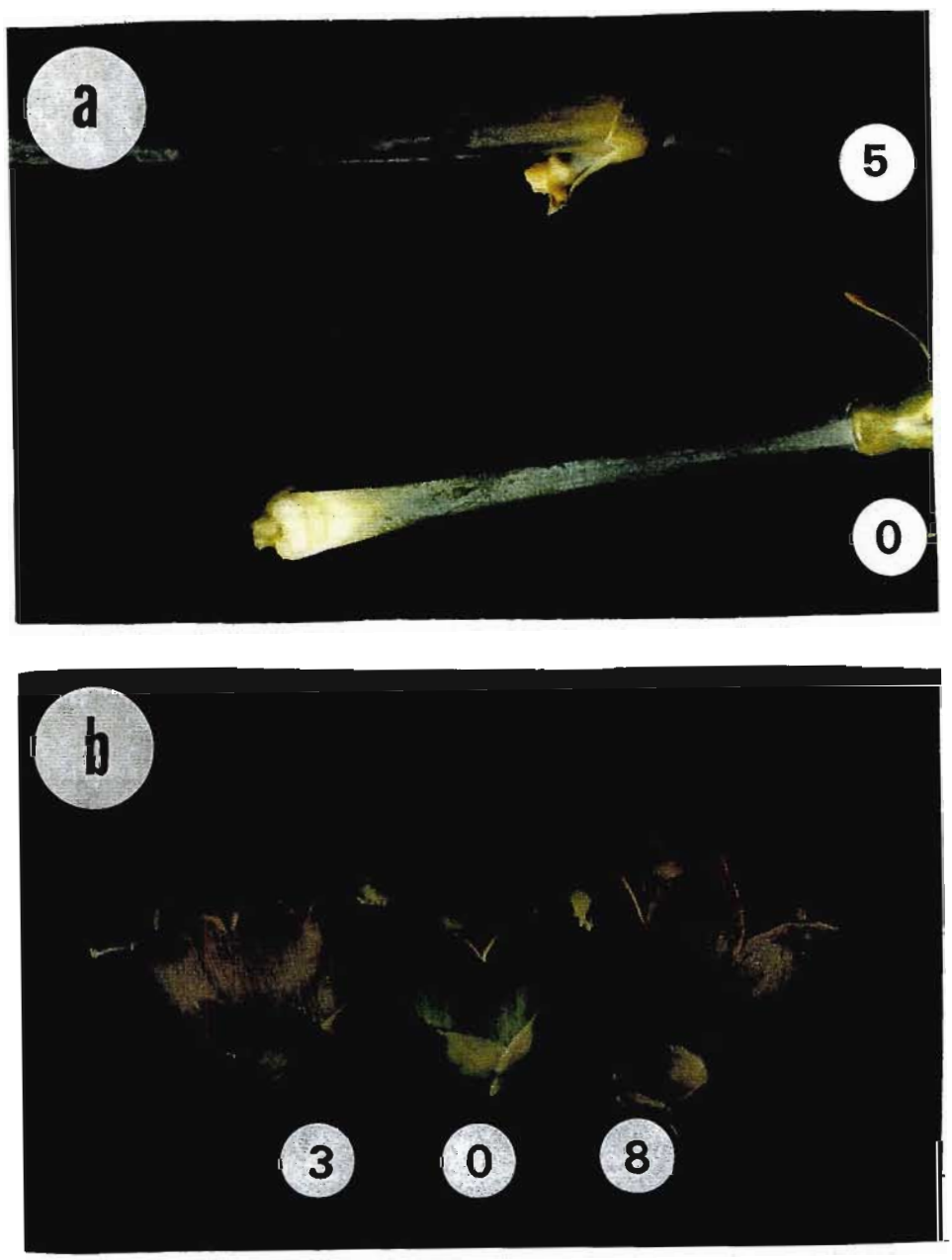


Plate 3.3. The appearance of stems (A) and calyxes (B) of cut carnation flowers treated with ethanol (Day 9), indicating the decrease in greenness. Text boxes indicate ethanol concentration.

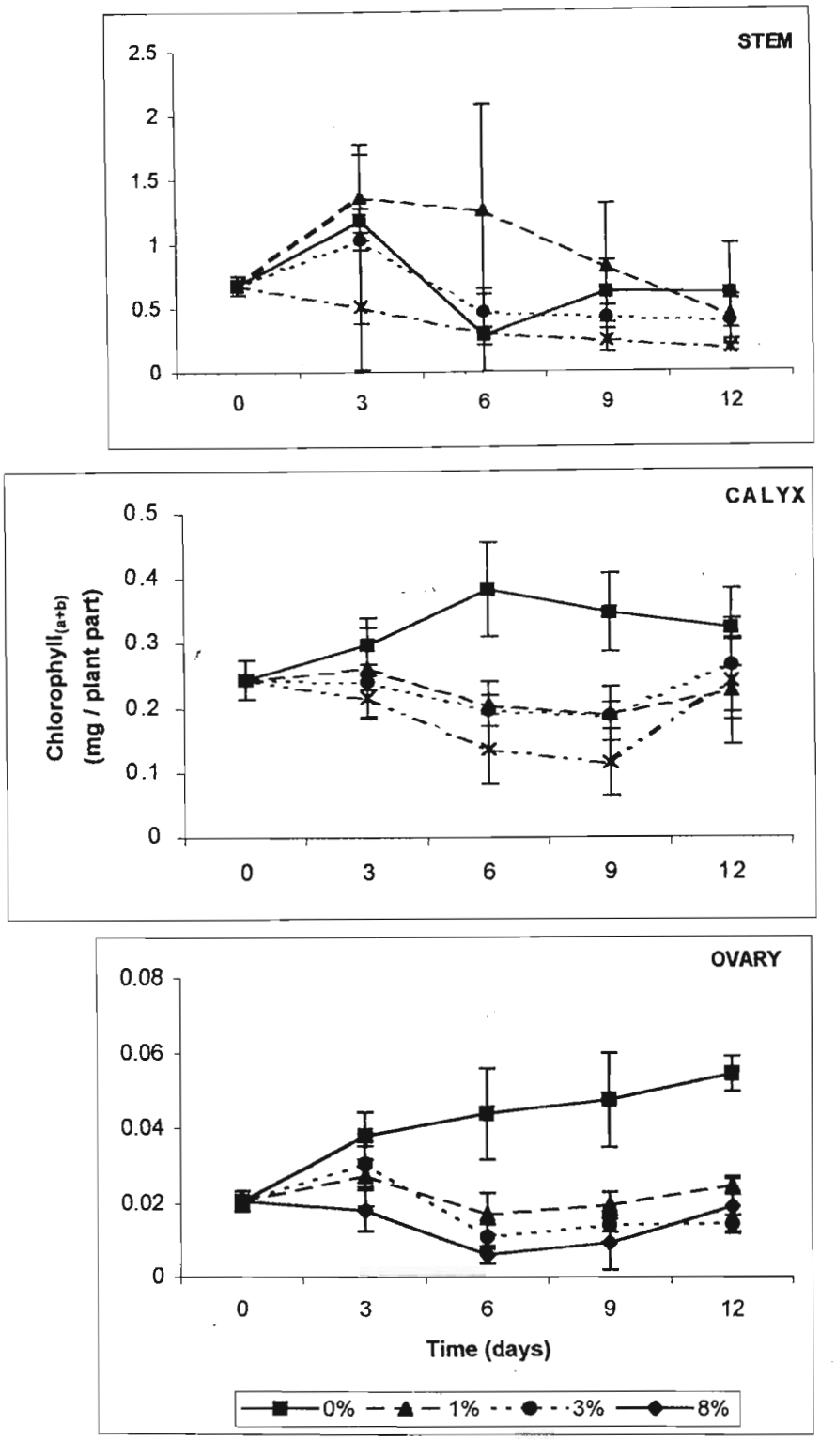


Figure 3.8. The effect of ethanol on the chlorophyll content of the stem, calyx and ovary. Note that the axes are not the same for each graph. Bars indicate standard errors.

content steadily increased throughout senescence, while this was distinctly inhibited in ethanol-treated flowers.

3.3.2. The effect of ethanol treatment on carbohydrate translocation and accumulation

Moisture content and dry matter results indicate that ethanol disrupts the source-sink activity of senescing carnation flowers. In order to determine how this occurs, it was necessary to consider the carbohydrate status of the flowers. Sugars were detected in petal, ovary and stem tissues of cut carnation flowers, and the change in their levels during ethanol-influenced senescence was recorded (FIGURE 3.9). The total carbohydrate content of the petals decreased substantially in both treated and control flowers. There was a significant difference between ethanol-treated flowers and the water control during the early stages of the experiment however. The rate at which the carbohydrate content of the petals decreased was disturbed by ethanol treatment, with levels decreasing at a much greater rate in treated flowers than the control flowers. The more ethanol applied to the flowers, the quicker the decrease in sugar. By the ninth day however, there was no longer any difference between treated and control petals. In the stem tissue of control flowers the carbohydrate content increased until the sixth day of treatment, after which it decreased. This was consistent with mass translocation of carbohydrates during senescence. In ethanol-treated flowers levels decreased sharply, and remained low for the rest of the experiment. This suggested a translocatory disruption, with the effect being more pronounced at higher ethanol concentrations. The most notable change in carbohydrate content was seen in the tissues of the ovary. In control ovaries there was a gradual increase in carbohydrate levels until the ninth day, corresponding with the sink activity of the organ. After this

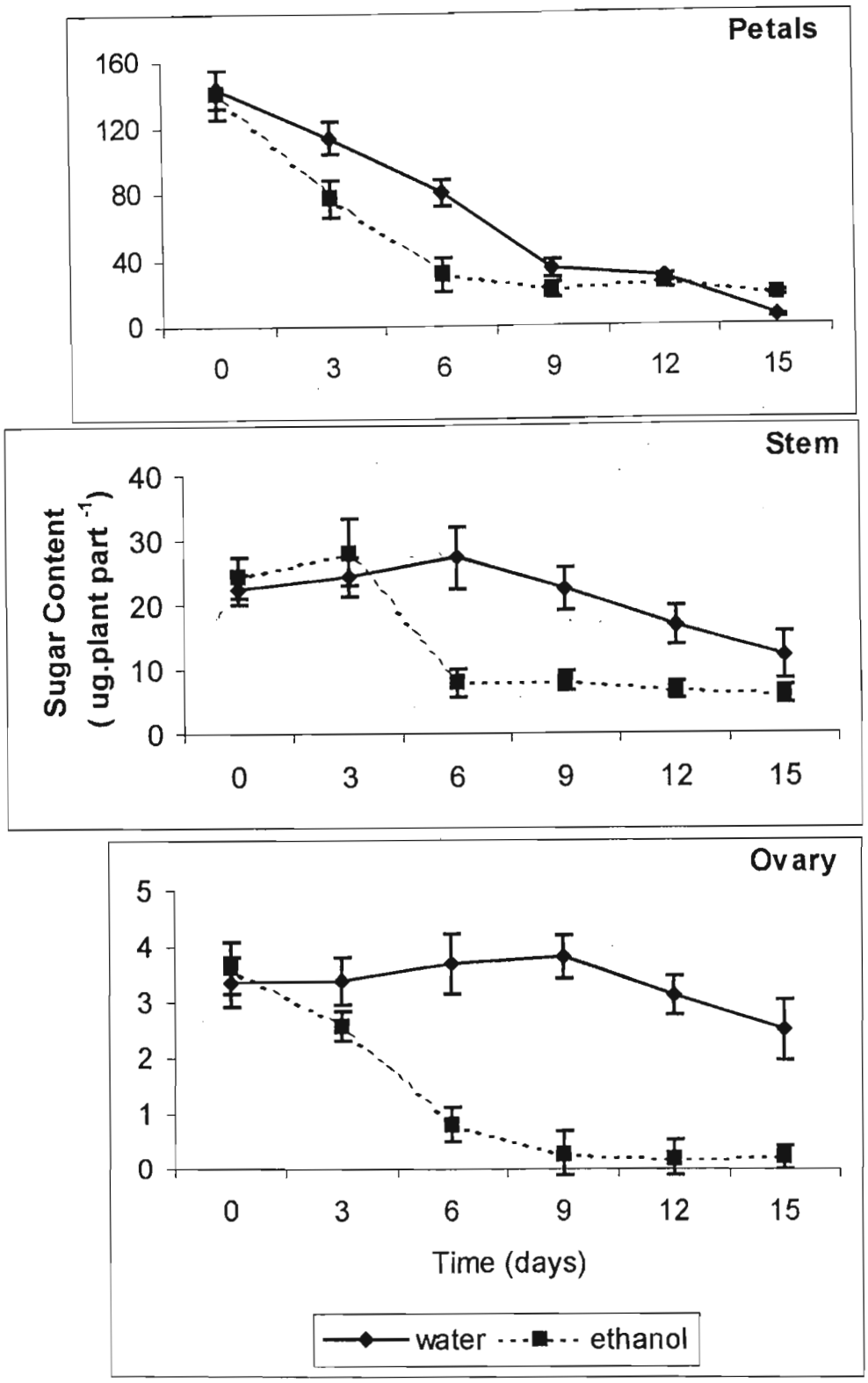


Figure 3.9. The effect of 3% ethanol on total carbohydrate content of cut carnation flowers. Note that the axes are not the same for each graph. Bars indicate standard errors.

levels decreased due to advanced senescence. In ethanol-treated flowers carbohydrate levels decreased constantly, and were significantly lower than the control flowers almost immediately after the onset of treatment. This indicated a potent inhibition of sink activity at all concentrations of ethanol.

Xylose, glucose, fructose, sorbose, galactose and mannose are all monosaccharides, which are products of photosynthesis and used to drive cellular respiration and metabolism. The trends for the monosaccharides were very similar to those seen in the total carbohydrate content. Levels in the petals decreased for both control and treated flowers, although more severely in the treated flowers (FIGURE 3.10). In the stems of control flowers, levels of monosaccharides decreased throughout the vase life. In the treated flowers levels decreased initially and remained reduced. In the ovaries of control flowers the monosaccharides increased significantly compared to levels in treated flowers, and remained increased until the fifteenth day of the experiment. In the treated flowers, levels dropped dramatically, and by sixth day there were only traces of monosaccharides remaining in the tissues.

Sucrose, a disaccharide, is a storage sugar, found in particularly high concentration in photosynthetic organs. Compared to monosaccharide levels, disaccharide levels were lower in the petal tissue due to the fact that these are not photosynthetic organs (FIGURE 3.11). Levels in both treated and untreated flowers decreased rapidly. In the control stems levels rose initially and then decreased, while there was an instant and dramatic decrease in all of the treated flowers. In the control ovaries the levels originally increased, however they decreased after the sixth day. It is possible that this was as a result of metabolism during the climacteric respiration

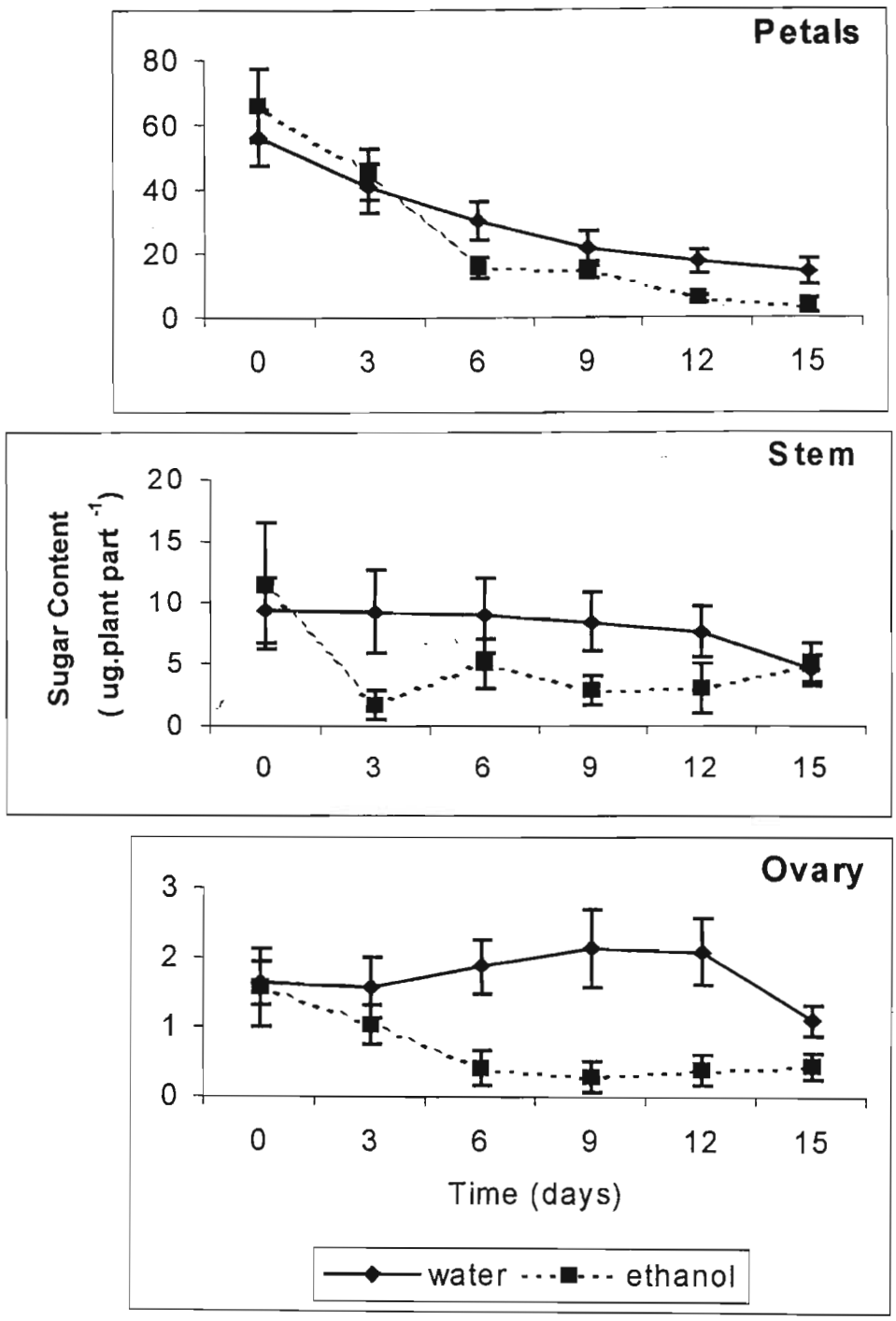


Figure 3.10. The effect of 3% ethanol on monosaccharide content of cut carnation flowers. Note that the axes are not the same for each graph. Bars indicate standard errors.

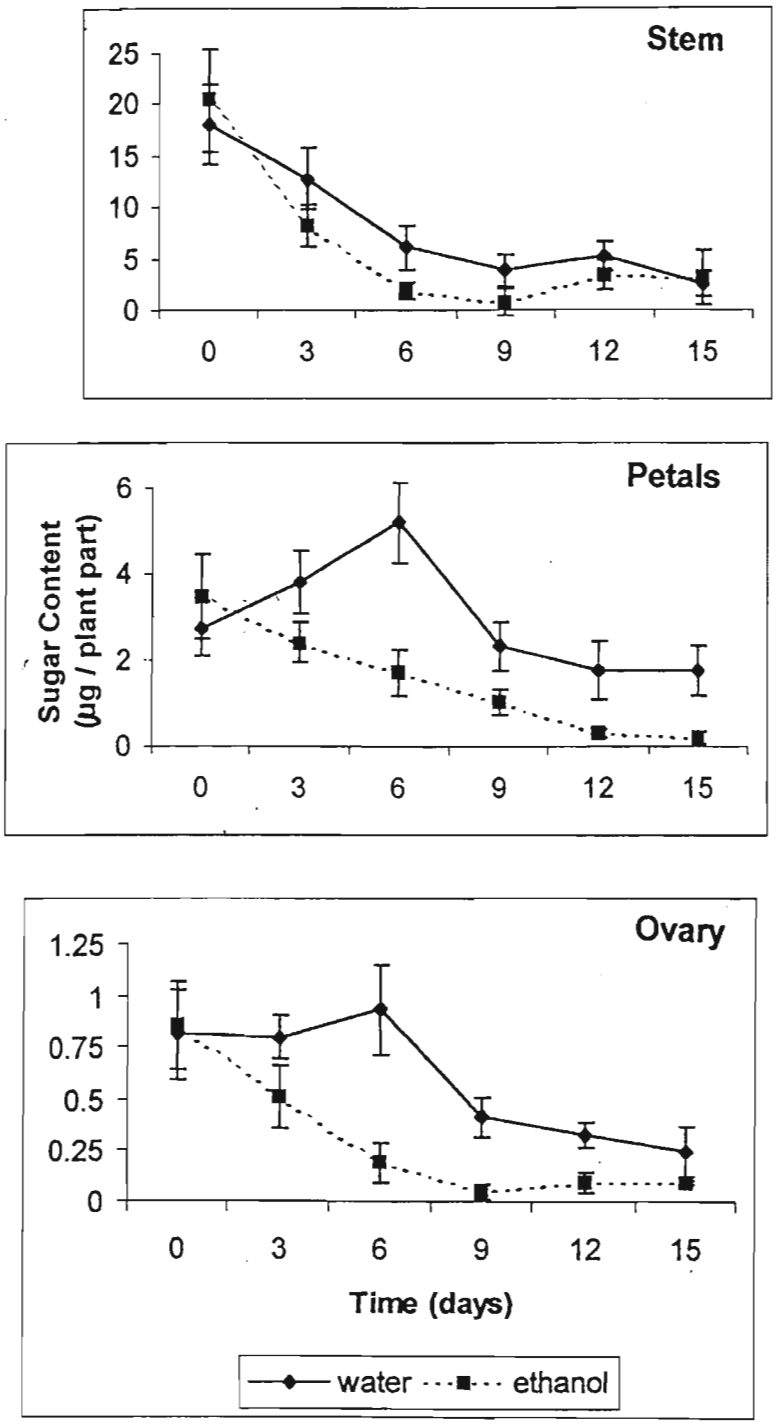


Figure 3.11: The effect of 3% ethanol on disaccharide content of cut carnation flowers. Note that the axes are not the same for each graph. Bars indicate standard errors.

increase. In the treated tissues levels once again decreased straight after the onset of ethanolic treatment.

Three sugar alcohols (inositol, mannitol and sorbitol) were detected in the tissues of senescing carnation flowers. They were less affected by ethanol treatment than the monosaccharides and disaccharides (FIGURE 3.12). In the petals there was a decrease in the ethanol-treated flowers compared to the water control on the third day, but by the sixth day levels in the water control had also decreased radically and no significant difference occurred. In the stems there was no difference between levels in treated and untreated flowers. Levels of sugar alcohols in the ovaries of control flowers increased until the ninth day, then decreased. In the treated flowers there was a sudden initial decrease in sugar alcohols in the ovary tissue, and levels remained very low until the end of the experiment.

In order to determine the effect of ethanol on the translocation of carbohydrates, ^{14}C sucrose was applied to a marked petal of both control and treated flowers and its movement monitored (FIGURE 3.13). The longevity of these flowers was different from the flowers used in previous experiments, with the water control being 50% senesced on day 5, and the ethanol-treated flowers on day 8. A fair amount of the ^{14}C was detected in the extract from the marked petal of the control flower, as well as the ethanol extract the petal was washed in, indicating that some of the ^{14}C was not absorbed by the flower and had remained on the surface. Even more ^{14}C was recorded in the marked petal and ethanol wash extracts of the treated flowers, and this was probably due not only to the ^{14}C sucrose not being absorbed, but also due to a lack of translocation within the system. Around 20% of the ^{14}C was recovered in the

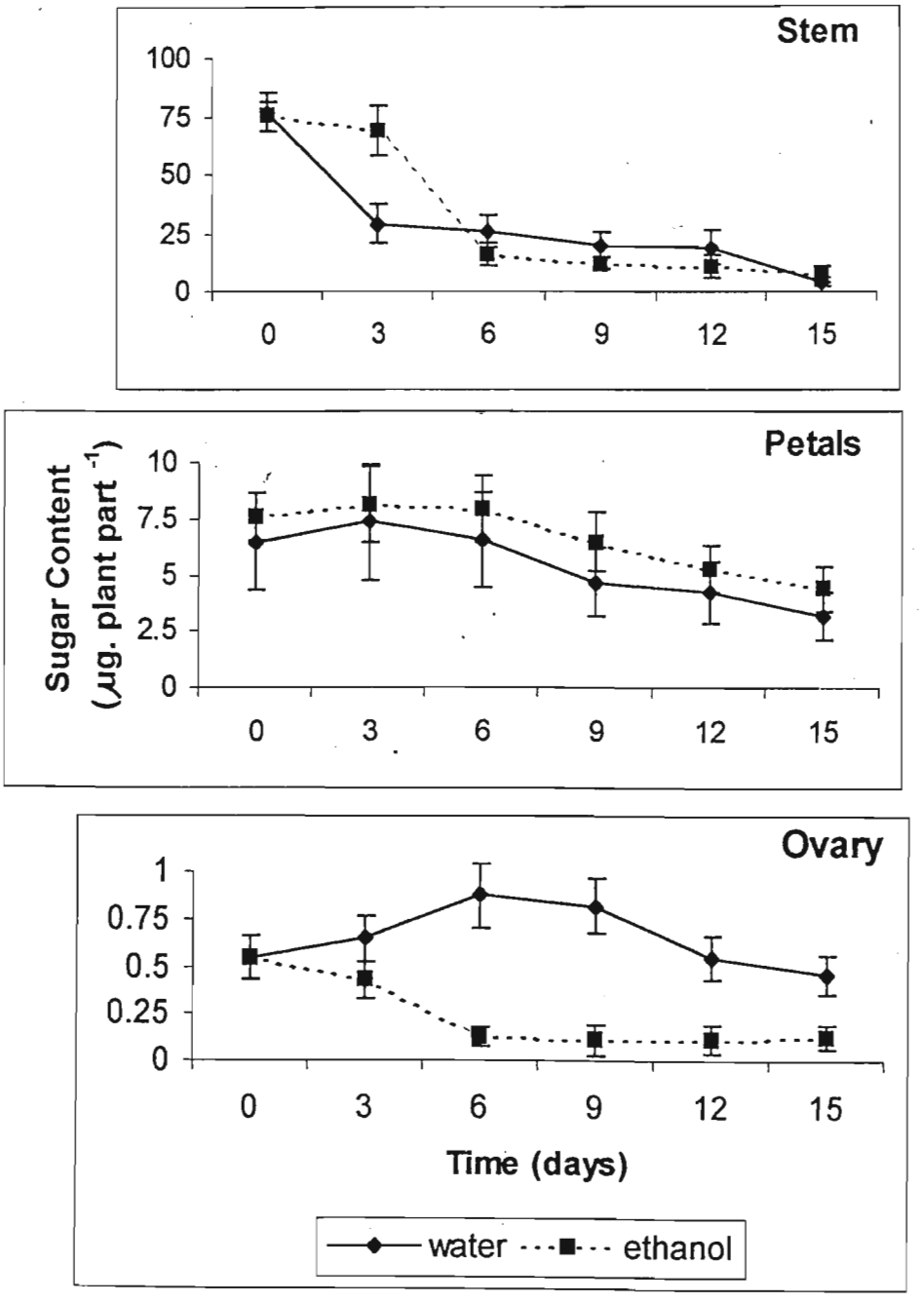


Figure 3.12. The effect of 3% ethanol on sugar alcohol (inositol, mannitol and sorbitol) levels of cut carnation flowers. Note that the axes are not the same for each graph. Bars indicate standard errors.

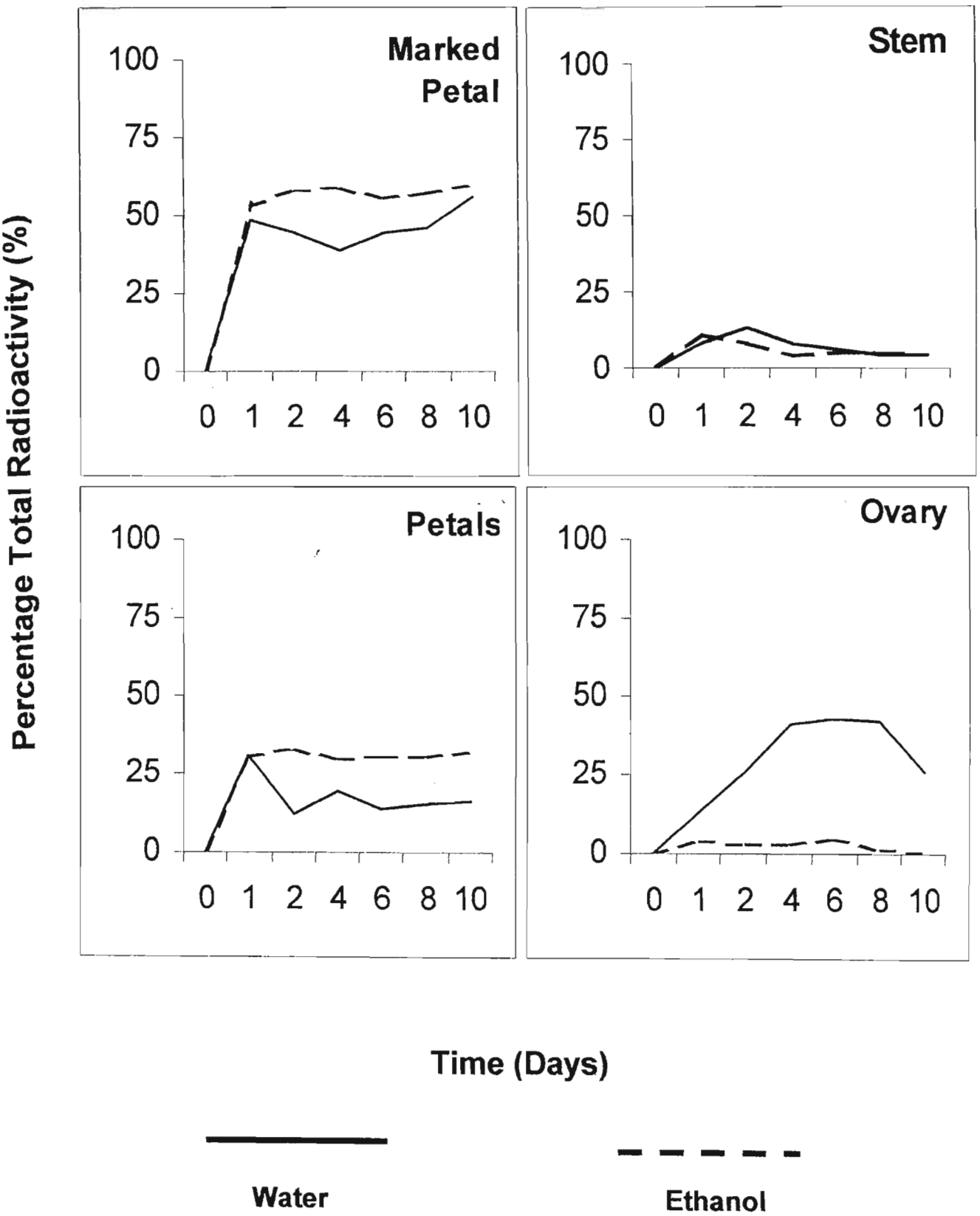


Figure 3.13. The percentage of the total applied ^{14}C sucrose recovered in the various organs.

other petals for the water control. Higher levels, around 40%, of the radioactivity were recovered in the petals of ethanol-treated flowers. Towards the end of the experiment up to 50% of the applied ^{14}C was recovered in the ovary tissues of the control flowers. This supported the work in the literature which stated that the ovary acted as a carbohydrate sink within senescing carnation flowers. In ethanol-treated flowers however, little to no ^{14}C was recovered in the ovary. This confirmed that the source sink activity of the flower had been interrupted by ethanol treatment. Levels of ^{14}C in the stem tissue of control flowers fluctuated, but were highest around the time of the greatest translocation of material from the petals. There was no difference between levels the stem regardless of the treatment.

3.3.3. The movement of applied ethanol

A pronounced effect of ethanol treatment is the destruction of the ovary as a carbohydrate sink, but are other organs effected by ethanol application? In order to try and trace the movement of applied ethanol, flowers were treated with ethanolic solution spiked with ^{14}C ethanol, and the amount of ^{14}C recovered in each organ of the flower was monitored. Results indicated the position of the ^{14}C only, however, and if the ethanol had been metabolised the results did not reflect this. Data was recorded 2, 4, 6, 8, 10 and 12 days after treatment began. No difference was detected between the percentage recovered on the different days, and the average percentage ^{14}C recovered in each organ throughout the experimental process was calculated (FIGURE 3.14.a.). Only 9% of the total recovered ^{14}C was found in the ovaries, while 86% of the ^{14}C was found to be in the petals, and the remaining 5% was in the stem/calyx complex. That such a large amount of the radioactivity was recovered in the petals indicated that the ovary was therefore not the only organ affected by ethanol treatment. While a huge

amount of the ^{14}C occurred in the petals, the petals as an organ are much larger than the ovary. When the amount of radioactivity found in each gram of plant tissue was considered (FIGURE 3.14.b) a different picture appeared. The amount of radioactivity per gram was seven times higher in the ovary than in the petals. Five percent of the radioactivity was recovered from the stem and calyx unit. This was to be expected, and was the only organ in which any sort of time pattern appeared, with levels being highest at the beginning of the experiment and the decreased. This was a result of the movement of the ^{14}C from the holding solution towards the head of the flower where the majority of the ^{14}C was recovered. Assuming no metabolism occurred, and the ^{14}C was still in the form of ethanol, it seemed as if the applied ethanol moved via the stem to the floral head, where it remained, in both the ovary and the petals.

3.3.4. The effect of ethanol treatment on the protein content of cut carnation flowers

Cells contain DNA, RNA and proteins such as enzymes and many other metabolic compounds. If these were interfered with, it would lead to the cessation of cellular metabolism and the functioning of the cell. The protein content of ethanol-treated cells was determined and compared to control flowers (FIGURE 3.15.a.). Total protein content of the petals of ethanol-treated flowers was much higher than in the water control over the entire experimental period. The S50 of the water control flowers occurred on the fifth day, explaining the sharp decline in the protein content after this time. The control flowers lived until the tenth day, and were still alive and healthy at this stage. These results were expressed as µg protein.plant part^{-1}. But the mass of the petals of the ethanol-treated flowers was substantially greater than that of the

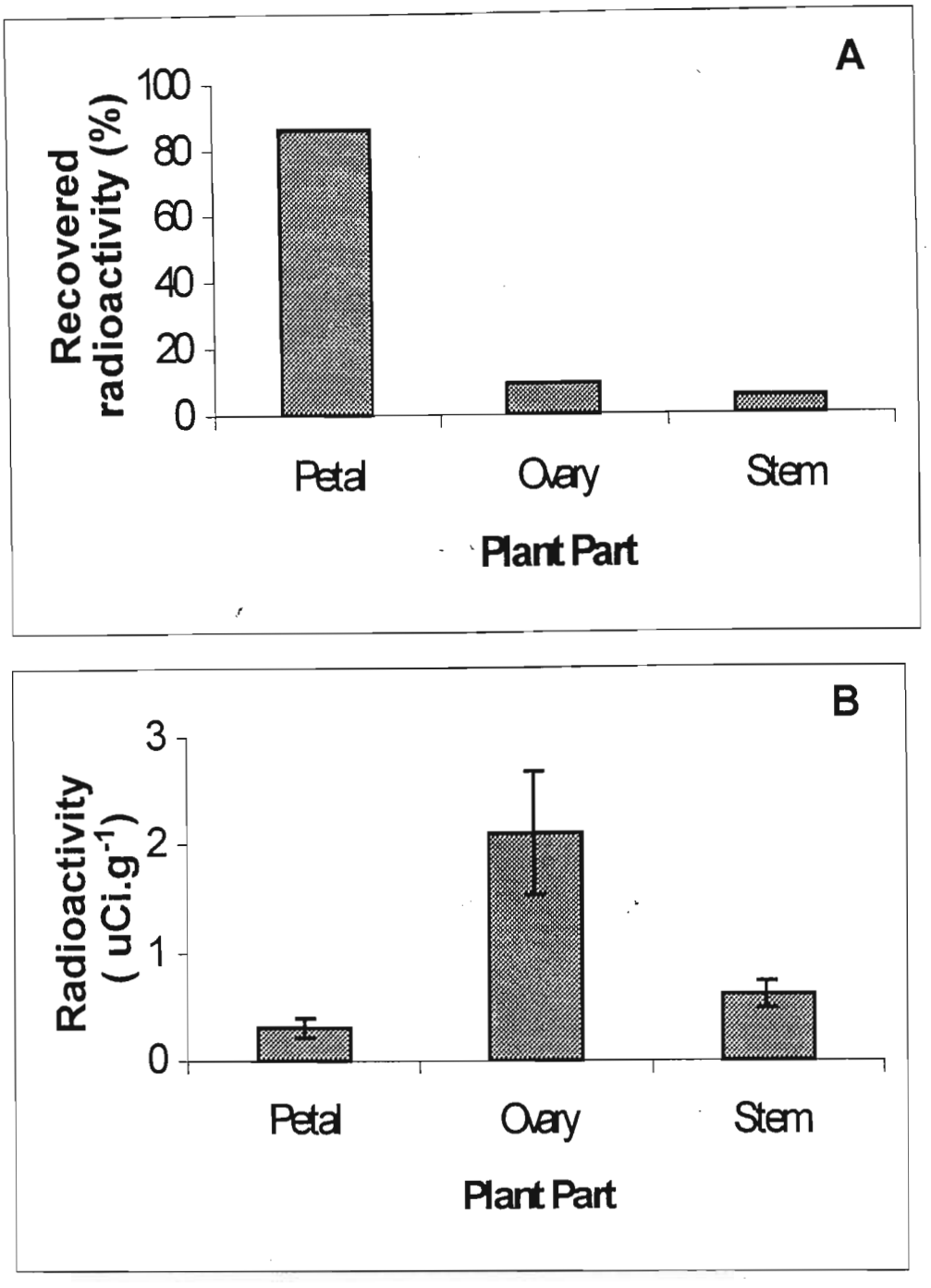


Figure 3.14. The amount of ^{14}C, applied as 3% ethanol, in organs of ethanol-treated flowers. A = the % of the applied radioactivity recovered in the organ. B = the amount of radioactivity recorded in each gram of the organ (µCi.g plant material^{-1}). Bars indicate standard errors.

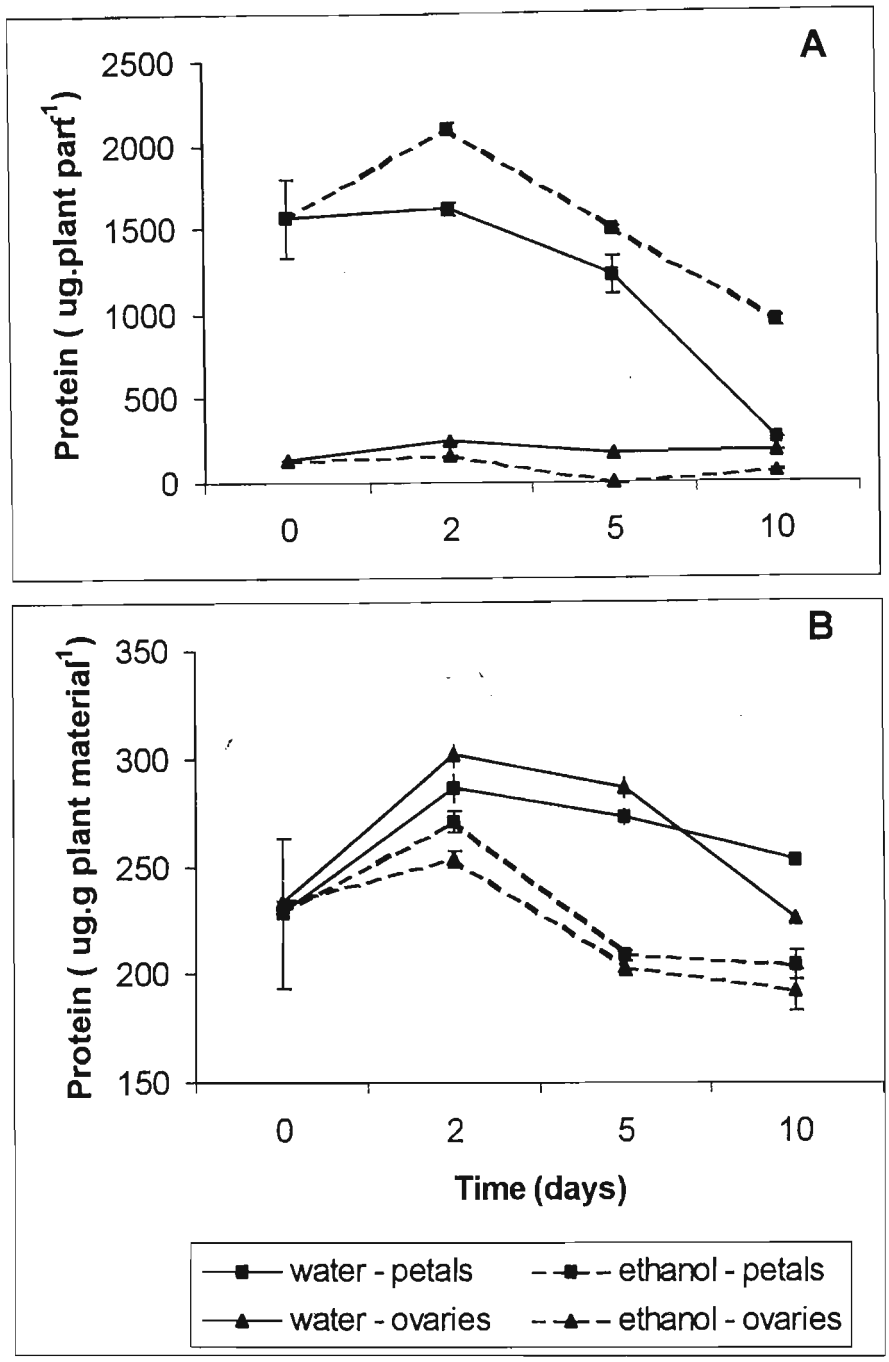


Figure 3.15. The effect of 3% ethanol on the protein content of petals and ovaries. A presents the results as mg.plant part^{-1} while B presents the results as mg.g plant material^{-1}. Bars indicate standard errors.

control flowers, as the control flowers senesced much sooner. When the same data were analysed as µg protein.g plant material^{-1} (FIGURE 3.15.b) a significant decrease in the total protein content of the ethanol-treated petals was observed when they were compared to the water controls. Regarding the ovary, total protein content of ethanol-treated flowers was significantly lower than the control ovaries when represented as mg protein.plant part^{-1} and mg.g plant material^{-1}. It therefore appeared that ethanol treatment resulted in a decrease in the protein content of the cells.

The recorded protein losses due to ethanol treatment could have been manifested in one of two ways. The ethanol treatment could have targeted specific enzymes systems within the cell, eradicating them entirely and resulted in decreased protein levels. The other option was a general loss of protein in cells, with ethanol affecting all systems equally. In order to determine which type of protein loss had occurred, SDS PAGE gels were run for protein extracted on day 2, 5 and 10. The gels separated 20 µg of protein, from the petals and ovaries of control and treated flowers, into proteins of similar sizes. The density of the band determined the amount of protein of each size grouping after staining, where darker areas contained more protein. The size of the proteins is measured as kilodaltons (kDa). On the second day of the experiment little difference could be seen between the bands of ethanol-treated and control petals or ovaries (FIGURE 3.16). On day 5 a major decrease could be seen in the smaller proteins (less than 26.6 kDa in size) of the treated petals and the ovaries (FIGURE 3.17.). This was more pronounced when samples from day 10 were run (FIGURE 3.18), and this time not only small proteins were effected, but all proteins. The lanes of the gel almost appeared smudged, indicating mass protein damage. These results suggested that a general loss in protein content occurred, although

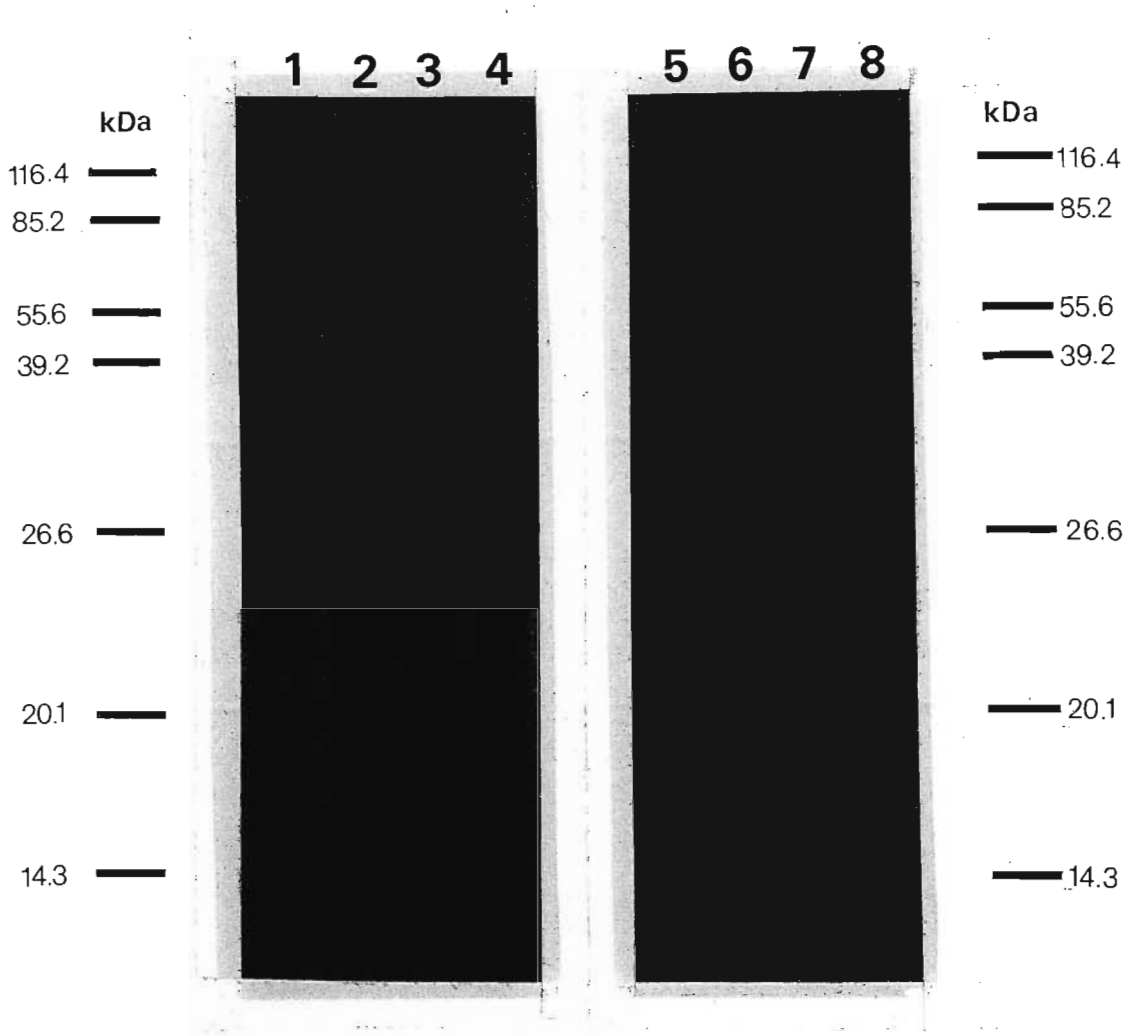


Figure 3.16. SDS-PAGE separation of proteins from control and ethanol-treated flowers on second day of treatment. Lanes 1 and 2 contain proteins extracts from control petals, lanes 3 and 4 contain proteins extracts from petals treated with 3% ethanol, lanes 5 and 6 contain proteins extracted from control ovaries and lanes 7 and 8 contain proteins extracted from ovaries treated with 3% ethanol. Little difference is observable between the banding of control and ethanol-treated extracts.

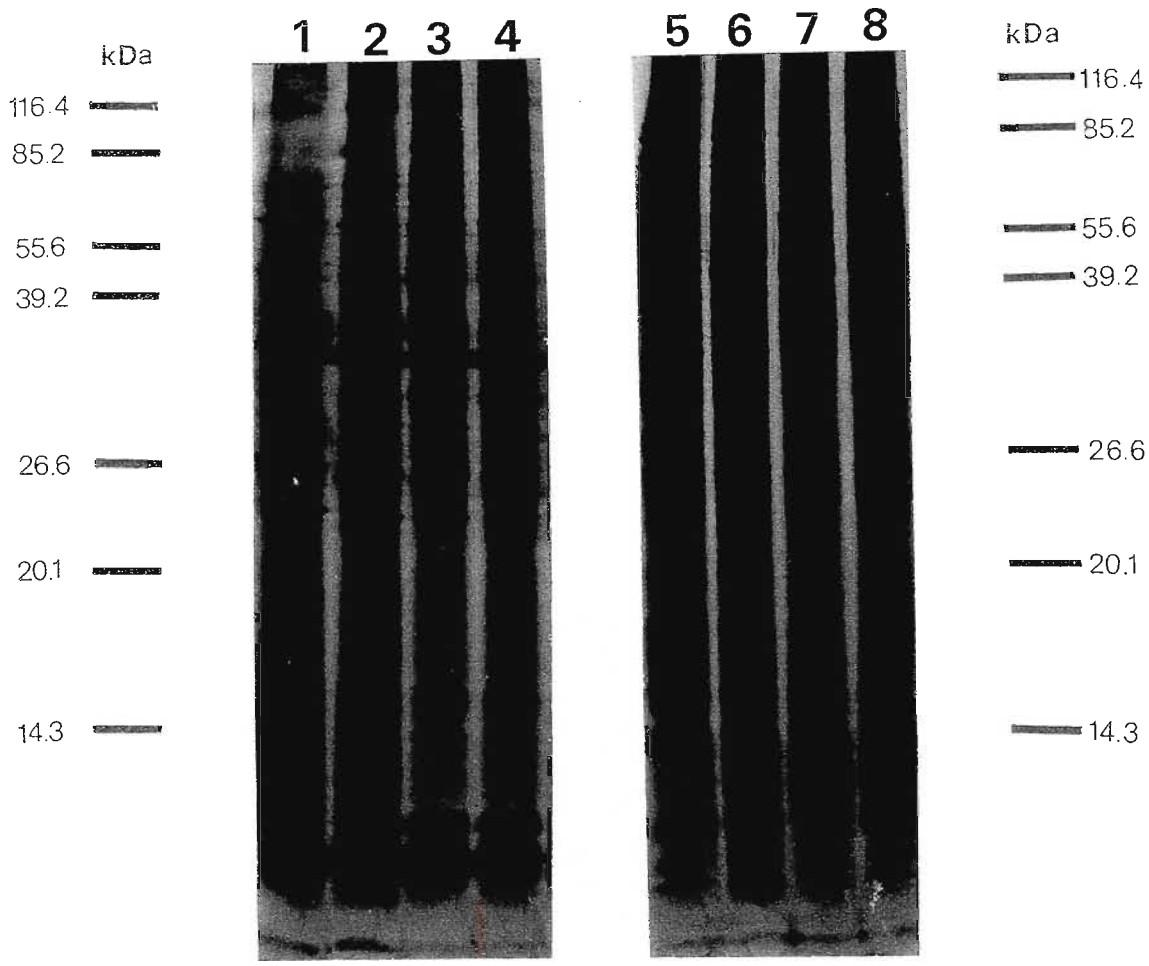


Figure 3.17. SDS-PAGE separation of proteins from control and ethanol-treated flowers on fifth day of treatment. Lanes 1 and 2 contain proteins extracts from control petals, lanes 3 and 4 contain proteins extracts from petals treated with 3% ethanol, lanes 5 and 6 contain proteins extracted from control ovaries and lanes 7 and 8 contain proteins extracted from ovaries treated with 3% ethanol. A loss of proteins smaller than 26.6 kDa can be seen in ethanol-treated petals and ovaries.

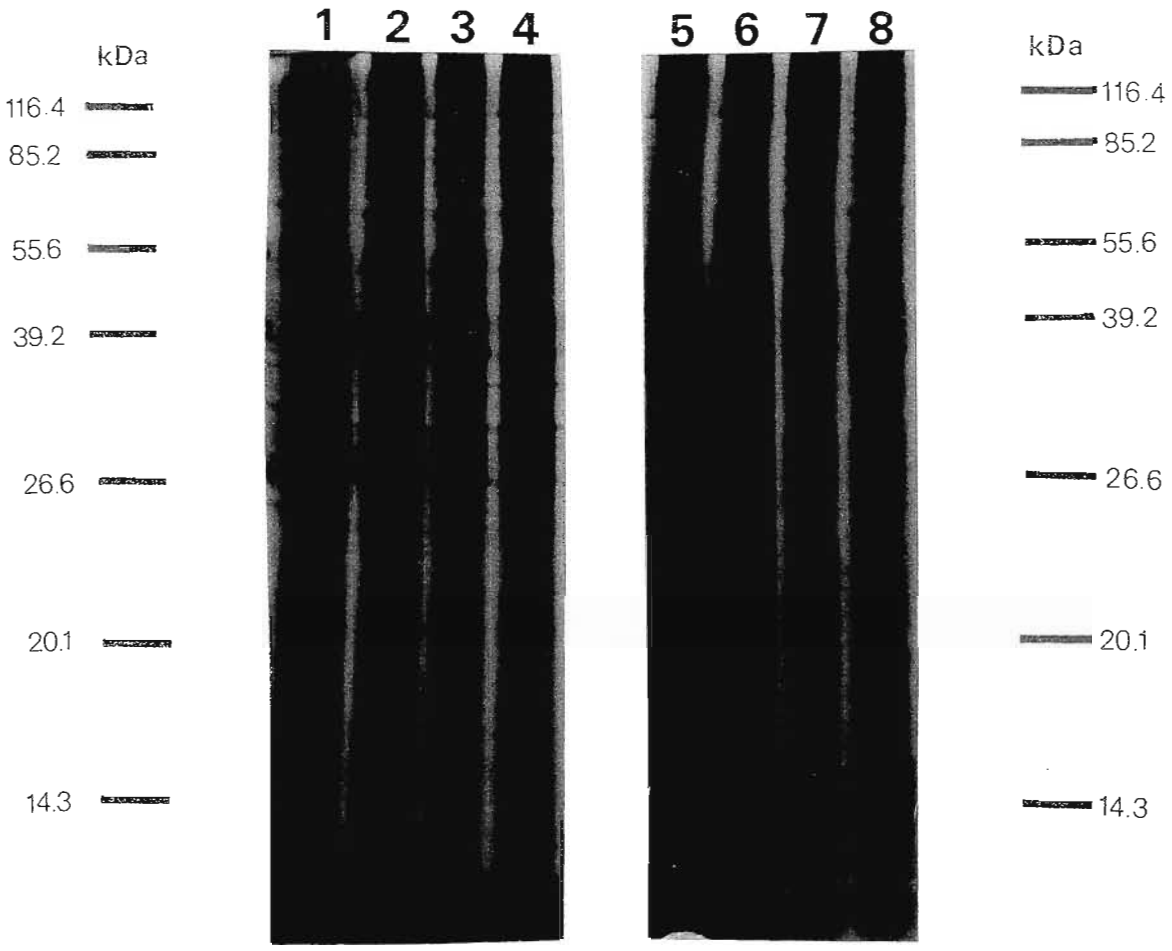


Figure 3.18. SDS-PAGE separation of proteins from control and ethanol-treated flowers on tenth day of treatment. Lanes 1 and 2 contain proteins extracts from control petals, lanes 3 and 4 contain proteins extracts from petals treated with 3% ethanol, lanes 5 and 6 contain proteins extracted from control ovaries and lanes 7 and 8 contain proteins extracted from ovaries treated with 3% ethanol. Major losses can be seen in treated extracts.

certain bands did remain dark in ethanol-treated flowers. These are indicated on the figures, and in general were proteins 39.3 kDa and larger in size. The reason these proteins were not affected is unclear.

3.3.5. The effect of ethanol treatment on ethylene formation

The effect of ethanol treatment of ethylene formation was determined in two ways: the effect of ethanol on ACC formation, as well as the activity of ACC oxidase, the enzyme responsible for the conversion of ACC to ethylene. Ethylene production was monitored in ethanol-treated flowers (FIGURE 3.19) and the ACC content (FIGURE 3.20) and ACC oxidase activity (FIGURE 3.21) of the petals and the ovaries of flowers were determined. The longevity of the flowers was measured (TABLE 3.8).

Table 3.8. The longevity of the flowers (from which the changes in ACC content and ACC oxidase activity was determined) treated with various concentrations of ethanol.

	Ethanol Concentration (%)					
	0	***1***	***3***	***5***	***8***	***10***
S50 (days)	5	7	10	8	7	6

In the control petals and ovaries a huge rise in ACC was seen on the first few days of the experiment, and ACC oxidase was 100% active. This was associated directly with the maximum production of ethylene seen on the third day. As a result of this ethylene formation, the control flowers were 50% senesced on the fifth day of the experiment. The treated flowers produced little to no ethylene. This was because ethanol treatment almost completely prevented the formation of ACC within the petal

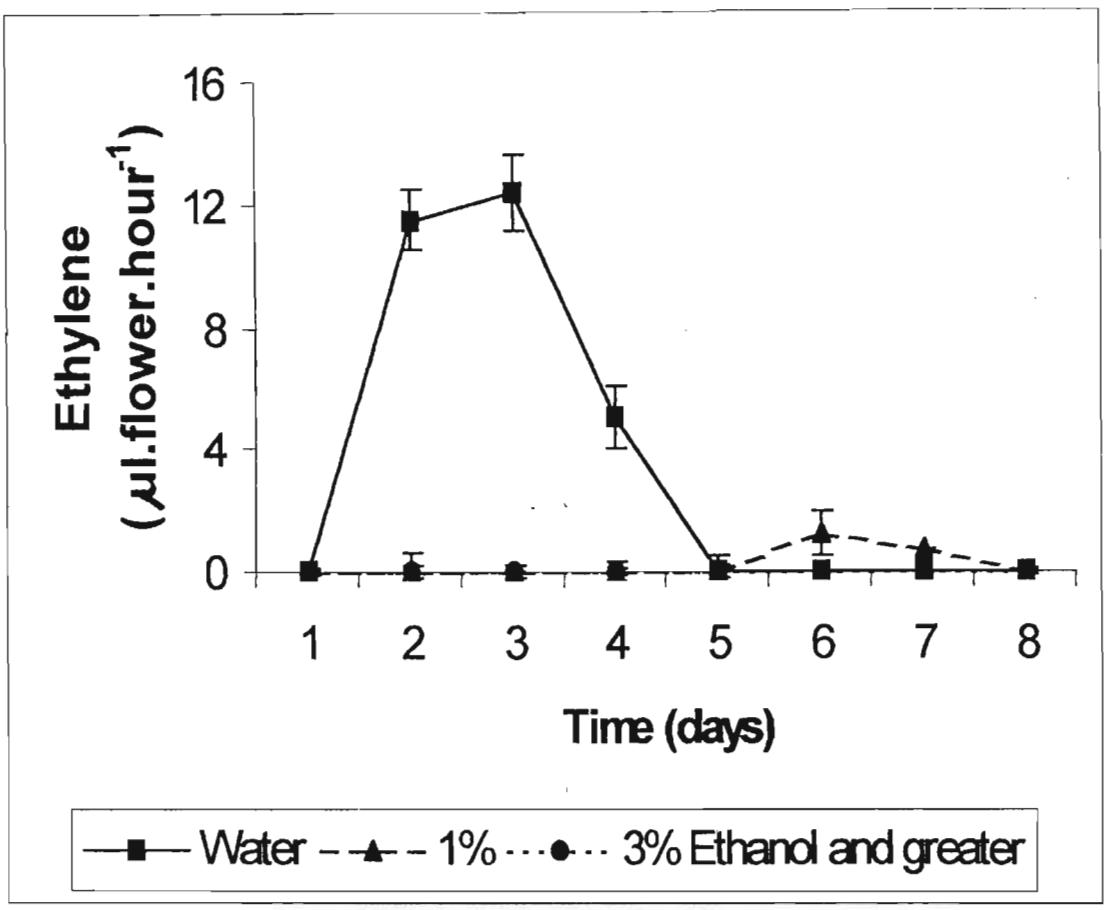


Figure 3.19. Levels of ethylene produced by the flowers used for ACC and ACC oxidase experiments. Bars indicate standard errors.

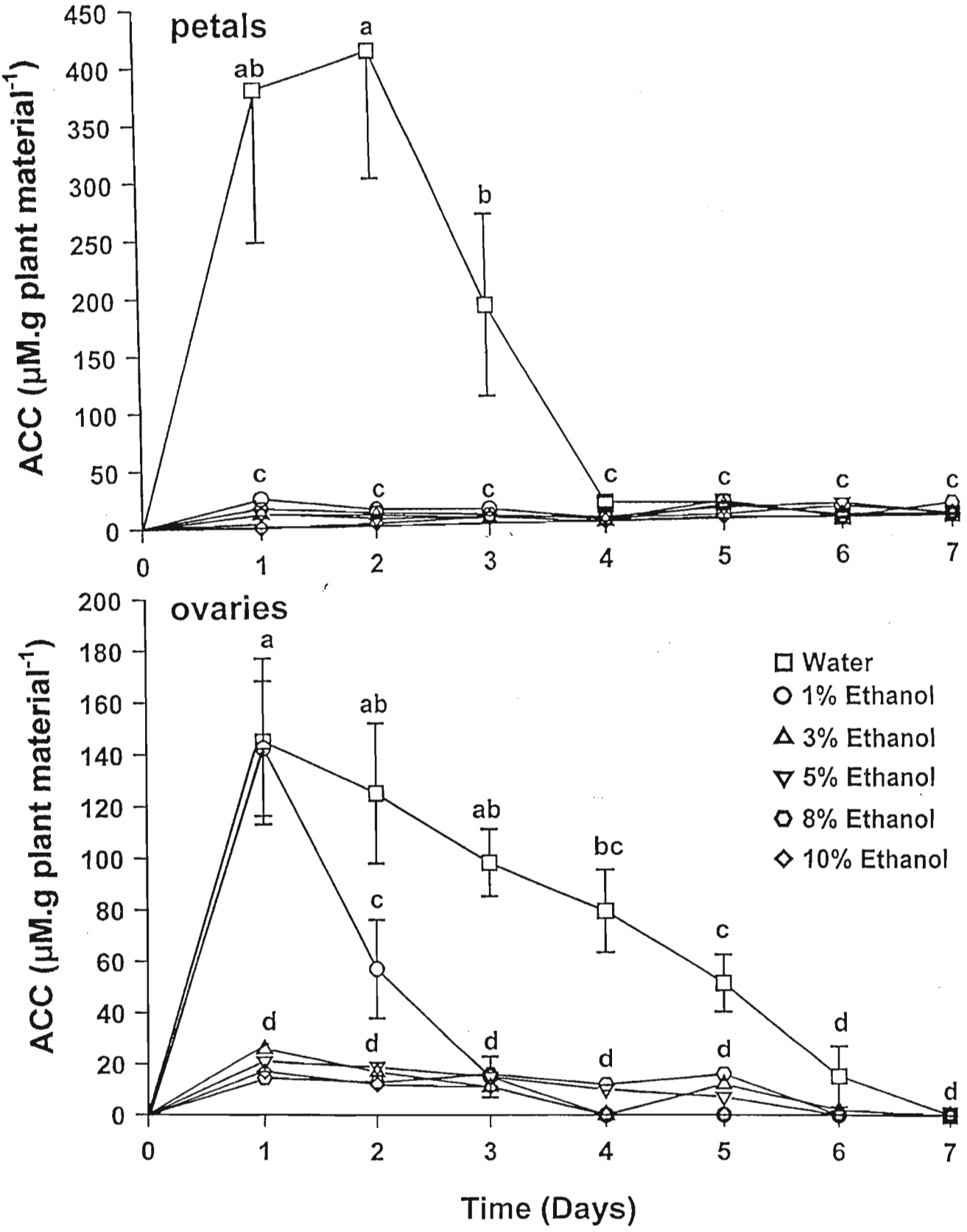


Figure 3.20. ACC levels in the petals and ovaries of treated and untreated cut carnation flowers. Bars indicate standard errors. Bars with the same letters are not significantly different from one another.

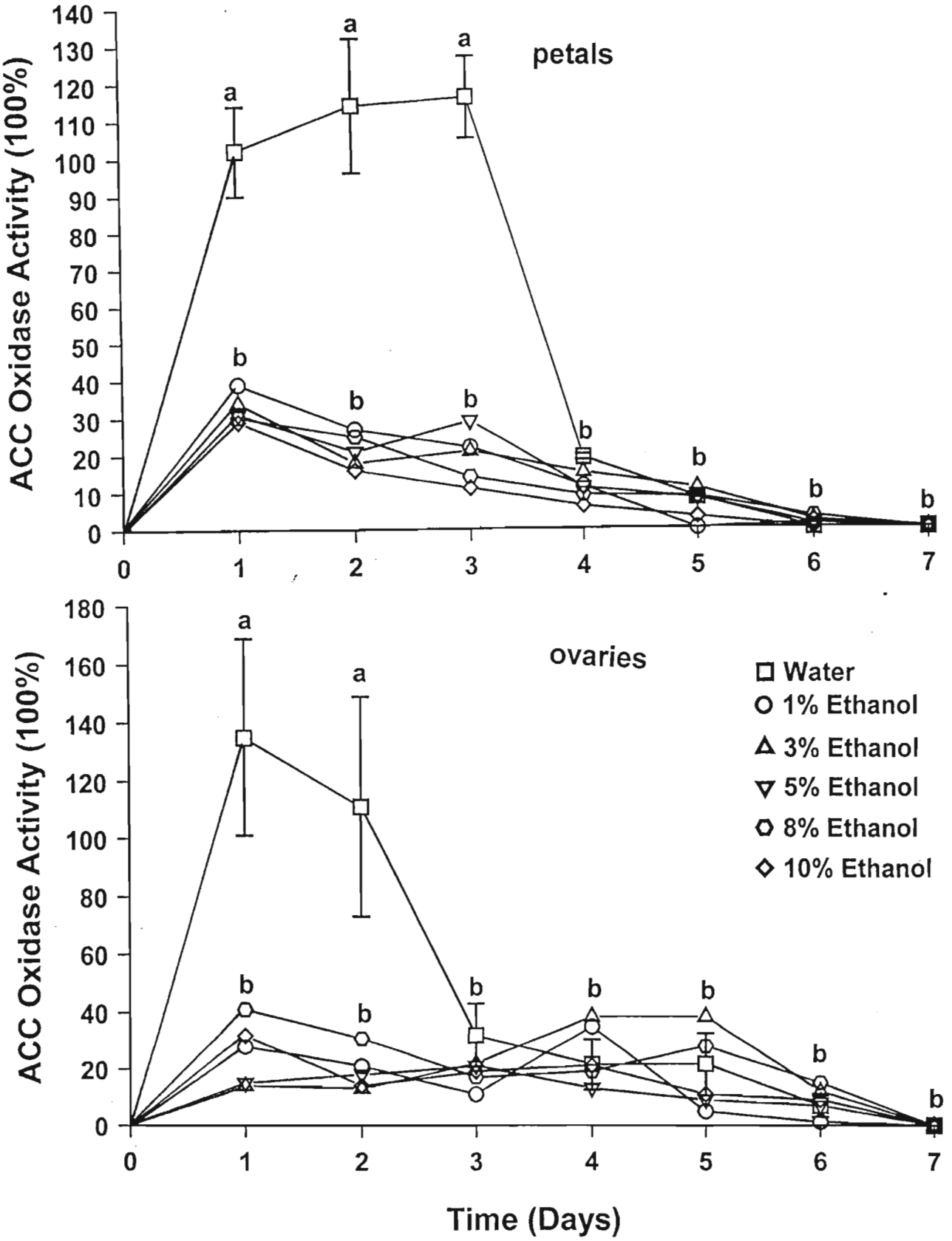


Figure 3.21. ACC oxidase activity in the petals and ovaries of treated and untreated cut carnation flowers. Bars indicate standard errors. Bars with the same letters are not significantly different from one another.

tissue of carnation flowers, at all concentrations. The activity of the enzyme ACC oxidase was also significantly reduced, to a maximum activity of 40% of its potential. In ovaries no ACC was recorded for 3% ethanol and higher. When 1% ethanol was applied to flowers, some ACC was produced in the ovaries. There was no significant production of ethylene by the flowers however. The ACC level in 1% tissues was significantly lower than control flowers.

The ACC oxidase activity in the ovaries was reduced for all concentrations of ethanol applied, again to about 40% activity. Therefore, in the flowers treated with 1% ethanol, some ACC was formed, but due to decreased ACC oxidase activity it was not converted to ethylene successfully. The decrease in ACC content and ACC oxidase activity successfully prevented ethylene formation in ethanol-treated flowers.

3.4. Discussion

The physiological effects of ethanol treatment on cut carnation flowers included the prevention of ovary development, petal browning instead of in-rolling, a loss of chlorophyll, the destruction of the ovary as a carbohydrate sink, the inhibition of ethylene formation and a decrease in the protein content of the cells.

In normally senescing carnation flowers the ovary acted as a carbohydrate sink. Carbohydrates were mobilised from the petals and translocated to the ovary. This resulted in the ovary gaining weight, and confirmed the results in the literature (NICHOLS 1977a; HALEVY and MAYAK, 1979; DIMALLA and VAN STADEN, 1980; DUCASSE and VAN STADEN, 1981; COOK and VAN STADEN 1983). At the same

time the chlorophyll content of the ovary increased, due to chloroplast growth (COOK and VAN STADEN, 1983; 1986). The increase in ovary dry weight and chlorophyll content has been widely attributed to ethylene formation. Ethylene is closely linked to carbohydrate movement, if not acting as a trigger (NICHOLS 1977a; CAMPRUBI and NICHOLS, 1979; COOK and VAN STADEN, 1983). The exact mechanism of ethylene's involvement is not known. Ethanol treatment prevented ethylene formation, and the accumulation of carbohydrates in the ovary. The moisture content and dry matter content of ethanol-treated ovaries decreased compared to the water controls. This proved to be due to the prevention of carbohydrate movement from the petals to the ovary. The prevention of mass carbohydrate mobilisation prevented the characteristic in-rolling of the petals. The ovary no longer appeared to act as a carbohydrate sink. When ^{14}C sucrose was applied to one of the petals, very little was detected in the ovary, indicating that translocation was disturbed, probably by the inhibition of ethylene formation. In fact, the carbohydrate content of both the ovary and the petals decreased during the senescence process. There was also a reduction in the chlorophyll content of the ovary and the stem during ethanol-influenced senescence. This was most likely due to the prevention of chloroplast development by ethanol treatment (COOK and VAN STADEN, 1983). The lack of photosynthetic organs would have prevented carbohydrates being produced in the cells, unlike in control ovaries, which continue to photosynthesise during the senescence process (COOK and VAN STADEN, 1983). Ethanol-treated flowers were shown to exhibit increased cellular respiration during senescence, although at a lesser rate than in control flowers (HEINS and BLAKELY, 1980; WU, ZACHARIAS, SALTVEIT and REID, 1992). It is likely that the decrease in carbohydrate content in both the ovary and the petals of ethanol-treated flowers was due to the carbohydrates being used for

respiration and other forms of cellular metabolism, and not being replaced. The overall loss of protein content due to ethanol treatment could also have effected the inhibition of carbohydrate translocation, possibly removing the required enzymes from the system.

Another system completely interfered with by ethanol treatment was the formation of ethylene. The formation of ACC and the action of ACC oxidase were both inhibited, preventing the formation of ethylene within the senescing tissues of the ethanol-treated flowers. This confirmed the results reported in the literature (HEINS and BLAKELY, 1980; WU, ZACHARIAS, SALTVEIT and REID, 1992). A possible reason for the inhibition of ACC formation in the tissues was that the activity of ACC synthase may have been prevented by the ethanol treatment. This was highly likely, as ethanol did result in a decrease in the total protein content of the tissue. Any ACC that was formed would most likely have been broken down within the cells for the same reason.

When the movement of ^{14}C ethanol was traced, ^{14}C was found in both the stem and the ovary of treated flowers. Although this did not necessarily mean that the ^{14}C was still in the form of ethanol, it definitely seemed that these were the organs most severely effected by ethanol treatment, both physically and in terms of functioning. The protein levels in these organs decreased after treatment had commenced. Ethylene formation, photosynthesis, carbohydrate translocation and growth are all processes of natural senescence, and all required proteins to occur. It is proposed that ethanol was transported to the ovary and petals of carnation flowers. Here it caused the destruction or denaturation of a wide range of proteins. Amongst these were many of the proteins required for the senescence process, including those required for ethylene formation.

By preventing this, the ethanol prevented the normal senescence of climacteric flowers. Among the processes effected were carbohydrate translocation and chloroplast development. Instead, the end of the vase life eventually occurred due to excessive browning of the petals. This occurred due to starvation of the flower, as the carbohydrates within the system were used up for the process of respiration, which still occurred, although in a reduced rate. When applied in high concentrations, ethanol caused negative effects within the system. The ovary and the stems became soft and weak, indicating large-scale damage to the cell wall and membranes. There was also a huge colour change in the tissue, possibly due to severe chloroplast damage, rather than just the inhibition of chloroplast growth. Therefore, a fine line existed between preventing the senescence-causing processes with ethanol treatment, thereby extending the vase life of the flowers and causing phytotoxic effects within the system which reduce the flowers longevity.

CHAPTER 4

ACETALDEHYDE IS THE CAUSAL AGENT OF ETHANOL-INDUCED CUT CARNATION FLOWER LONGEVITY INCREASES

4.1. Introduction

Ethanol prevents cut carnation flower senescence (HEINS 1980; HEINS and BLAKELY, 1980; WU, ZACHARIAS, SALTVEIT and REID, 1992). It also prevents the ripening of tomato fruits (KELLY and SALTVEIT, 1988; SALVEIT and MENCARELLI, 1988). Much has already been discussed about the inhibition of flower senescence by ethanol (Chapters 2 and 3), particularly the inhibition of ethylene formation and the sink activity of the ovary. Ethanol does not only halt the senescence of cut carnation flowers however, it has also prevents the ripening of tomato fruit, even in the presence of ethylene. This can result in the tomatoes having a mottled appearance. Both methanol and n-propanol have been tested to see if they have a similar effect on tomato fruit. While methanol had no delaying effect on tomato fruit ripening, n-propanol had an inhibiting effect, slightly stronger than that of ethanol, with no negative effects on fruit appearance (KELLY and SALTVEIT, 1988). This disagrees with the effects of other alcohols when tested on cut carnation flowers (WU, ZACHARIAS, SALTVEIT and REID, 1992). However, n-Propanol did lead to much higher ion leakage levels than ethanol, and resulted in damage to the fruit (SALTVEIT, 1989). Ethanol vapours have also been shown to significantly reduce the ripening of pericarp discs, slices and whole tomatoes (SALTVEIT and MENCARELLI, 1988). The ripening inhibiting effect of

ethanol is not restricted to mature green fruit, but is also seen in pink and even red tomatoes, with no associated loss in fruit quality upon ripening (SALTVEIT and SHARAF, 1992). Even in very mature fruits, that had already passed the breaker-turning stage and begun climacteric ethylene production, ripening was halted by ethanol treatment (SALTVEIT and MENCARELLI, 1988). As the ethanol concentration increased, the ripening of the fruit decreased proportionally. If ethanol levels within the tissue become too high however, fruit ripening remains inhibited (SALTVEIT and MENCARELLI, 1988). The effect of an anaerobic environment, as opposed to an ethanol treatment, also inhibited fruit ripening (KELLY and SALTVEIT, 1988).

While studying the ethanol-induced injuries that occurred in carrot cells, it was determined that observed toxic effects of ethanol were in fact due to acetaldehyde. The alcohol dehydrogenase inhibitor 4-methyl pyrazole (4-MP) prevents the conversion of ethanol to acetaldehyde. The inhibitor 4-Indol pyrazole (4-IP) prevents the opposite reaction. When 4-MP was introduced to carrot cells, in combination with ethanol, no injury followed (PERATA and ALPI, 1991). When ethanol was applied to tomatoes, in the presence of 4-MP, the normal inhibition of ripening caused by ethanol treatment was not seen. It therefore appeared that the conversion of ethanol to acetaldehyde was essential for the inhibition of ripening, and that acetaldehyde was the causal agent responsible for ethanol-induced inhibition of tomato fruit ripening (BEAULIEU, PEISER and SALTVEIT, 1997).

The aim of this study was to determine whether acetaldehyde is present in ethanol-treated cut carnation flowers, where it occurs, the effect of ethanol and acetaldehyde on the activity of alcohol dehydrogenase, and if the application of

acetaldehyde leads to any resultant vase life increases.

4.2. Materials and methods

4.2.1. Atmospheric ethanol and acetaldehyde

Flowers treated with ethanol had a distinct odour not detectable in control flowers. This indicated that the flowers were releasing either ethanol or a by-product into the surrounding atmosphere. Carnations are climacteric flowers, and the standard procedure used to measure atmospheric ethylene production is to place flowers in individual glass jars for a period of one hour, after which a gas sample of the head-space of the jar is subjected to gas chromatography. A technique very similar to this was employed to determine the presence of atmospheric ethanol and acetaldehyde in 3% ethanol-treated and control flowers. Three flowers from each different treatment were enclosed in 1ℓ glass jars for a period of 2 h. After this a 1 mℓ sample of the head space was removed and analysed using the gas chromatography techniques outlined by HEINS, 1980. A Varian 3300 gas chromatograph fitted with a flame ionisation detector was used. The column was 183 cm by 0.3 cm, packed with 80 - 100 mesh poropak Q. The carrier gas was nitrogen, flowing at a rate of 60 mℓ.min^{-1}. Results were detected on a Hewlett-Packard integrator. A representative chromatogram is available in APPENDIX A. Data were recorded every two days, with flowers being discarded once they had been used for measurement.

4.2.2. Ethanol and acetaldehyde within tissue

In order to determine whether there were any significant changes in the levels of ethanol and other volatile compounds in the tissue after 3% ethanol treatment, it was necessary to be able to determine the levels of such compounds within plant material. This was done by following the extraction technique outlined by HEINS, 1980. Flowers were harvested every two days, with plant material being weighed and then ground after flash freezing in liquid nitrogen. The material was not dried in order to prevent loss of ethanol through evaporation. A 100 µg ground sample was weighed out, and extracted in 10 mℓ double distilled water for 24 h. Solutions were centrifuged in a Beckman centrifuge for 15 min at 10 000 g. Of the resultant supernatant, 3 µℓ was subjected to gas chromatography as described in Section 4.2.1.

4.2.3. Acetaldehyde treatments

Acetaldehyde solutions were prepared using fresh redistilled acetaldehyde, diluted with distilled water. These solutions were replaced every two days in order to prevent any evaporation effect. For all experiments, a distilled water control was provided.

4.2.4. 4-Methylpyrazole and 4-iodopyrazole alcohol dehydrogenase inhibitors

Pyrazole, and the more active 4-substituted pyrazole 4-methyl pyrazole (4-MP) inhibited both *in vivo* and *in vitro* oxidation of ethanol by horse liver alcohol dehydrogenase (ADH) (THEORELL, YONETANI and SJÖBERG 1969). 4-methyl pyrazole and other 4 substituted pyrazoles, such as 4-iodopyrazole (4-IP) are now

commonly used as potent inhibitors of ADH activity. 4-Methyl pyrazole prevents the conversion of ethanol to acetaldehyde (PERATA and ALPI, 1991) and 4-IP prevents the conversion of acetaldehyde to ethanol. A range of 4-MP concentrations, from 1 mM.ℓ^{-1} to 100 mM.ℓ^{-1} was tested on cut carnations. No negative effects resulted at high concentrations, and the compound was effective at low concentrations. Therefore 10 mℓ of 10 mM.ℓ^{-1} 4-MP was pulsed into flowers, either with distilled water or 3% ethanol. Once all 10 mℓ of the inhibitor had been taken up, the flowers were placed in holding solutions of either distilled water or 3% ethanol. Flowers treated with 4-IP were pulsed with 10 mℓ of 20 mM.ℓ^{-1} 4-IP (as per BEAULIEU, PEISER and SALTVEIT 1997). Once all the solution had been taken up, the flowers were placed in holding solutions containing either 2% acetaldehyde or distilled water.

4.2.5. Alcohol dehydrogenase activity

The amount of alcohol dehydrogenase (ADH) in ethanol- and acetaldehyde-treated cut carnation flower tissue was determined using a simple assay. When ADH reduces acetaldehyde to ethanol, a molecule of nicotinamide adenine dinucleotide phosphate (NADH) is oxidised to nicotinamide adenine dinucleotide (NAD^+). ADH is a multidirectional enzyme, and is also capable of oxidising ethanol to acetaldehyde, in which case NAD^+ is reduced to NADH. The activity of ADH in the tissues in either direction could therefore be monitored by measuring the increase or decrease in NADH levels in the tissue (KATO-NOGUCHI, 2000).

For measuring ADH activity in the direction of acetaldehyde to ethanol, 1mℓ

assay mixture was placed in a 1.5 mℓ Eppendorf tube. The mixture consisted of 85 mM MES (pH 6.5), 0.9 mM NADH and 100 µℓ plant extract. The reaction was initiated by added 5 mM acetaldehyde to act as a substrate for the reaction. The Eppendorf tube was sealed, and incubated at 30°C for 15 min, after which the sample was shaken and poured into a quartz curvette. The entrance of the curvette was sealed with parafilm to prevent evaporation. NADH was detected spectrophotometrically at 340 nm. The greater the amount of ADH in the sample, the less NADH will be present in the sample. Using an ADH standard, a concentration curve for absorbance and units enzyme was constructed (FIGURE 4.1). One unit of ADH converted 1 µM acetaldehyde to ethanol . min^{-1}, so the reading was divided by 15 to get the result as µM . $minute^{-1}$. ADH is a protein, and so it was also necessary to determine the amount of protein in the sample using the Bradford assay described in 3.2.8. The activity of ADH was expressed as µM . min^{-1} . µg $protein^{-1}$ (HANSON and JACOBSON, 1984; HANSON, JACOBSON and ZWAR, 1984). The dual action of ADH in both directions is a pH based phenomenon. In order to determine the activity of ADH in the opposite direction, (ethanol to acetaldehyde) a similar assay was employed. However, this time the assay mixture was made from 8.5 mM MES buffer at a pH of 8.0. The NADH in the sample was replaced with 0.9 mM NAD^+, and ethanol, rather than acetaldehyde, was required to initiate the reaction. The results were once again measured spectrophotometrically. A standard curve for absorbance was constructed (FIGURE 4.2.). This had a similar gradient as the curve for ADH in the opposite direction, however, as the activity of ADH increased in this case the amount of NADH in the sample increased. It was again necessary to determine the protein content of the samples, and results are expressed as µM . min^{-1} . µg $protein^{-1}$. g fresh $weight^{-1}$ (HANSON and JACOBSON, 1984).

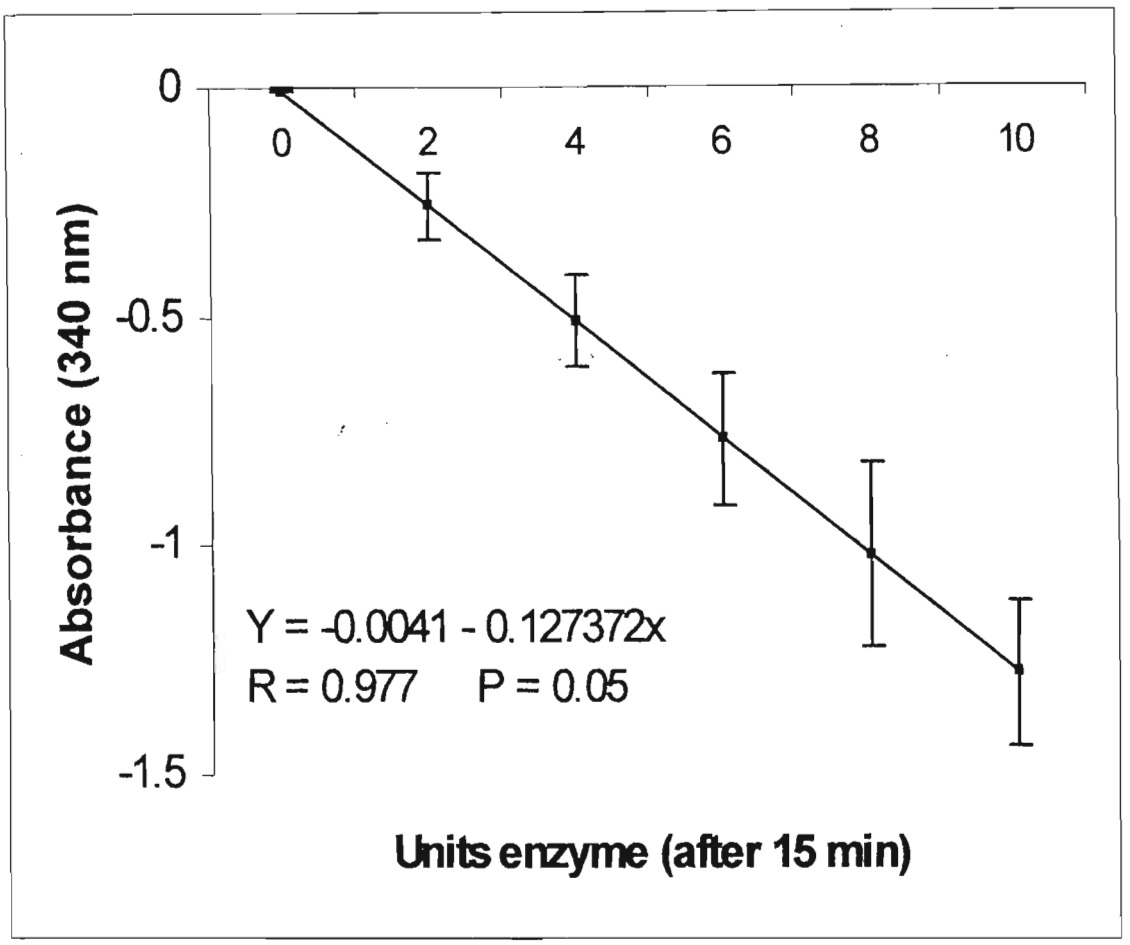


Figure 4.1. Standard alcohol dehydrogenase concentration curve, (in the acetaldehyde → ethanol direction) as detected spectrophotometrically. Bars indicate the standard errors.

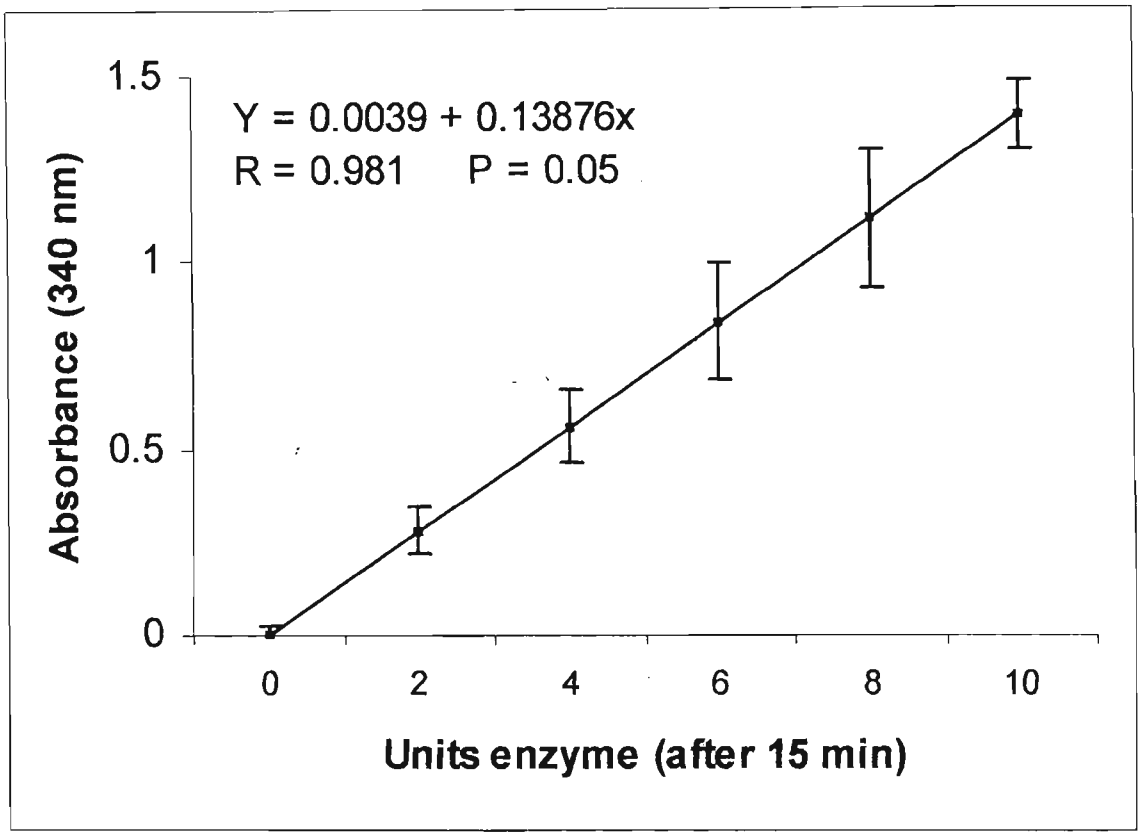


Figure 4.2. Standard alcohol dehydrogenase standard curve (in the ethanol → acetaldehyde direction), as detected spectrophotometrically. Bars indicate standard errors.

There are two buffers than can be used to extract ADH: TRIS (HANSON, JACOBSEN and ZWAR, 1984; KATO-NOGUCHI, 2000) and MES (KE, YANIA, MATEOS and KADER, 1994; KATO-NOGUCHI and WATADA, 1997). A number of additives have been included in these buffers to optimise extraction, including DTT, sodium ascorbate (Na-asc), BSA, glycerol, PVP and PVPP. It was necessary to perform trials to determine the best extraction buffer. Firstly, the original buffers from the literature were tested, when 250 mg of material from the same plant was extracted using 4 different buffers. TRIS A consisted of 100 mM TRIS buffer containing 10 mM DTT, 10 mM Na-asc, 2 mM BSA and 5% glycerol (KATO-NOGUCHI, 2000). TRIS B contained only 100 mM TRIS and 10 mM DTT (HANSON, JACOBSEN and ZWAR, 1984). Both MES-based buffers contained 100 mM MES and 2 mM DTT, but MES A contained 1% soluble PVP (KE, YANIA, MATEOS and KADER, 1994) and MES B contained the insoluble PVPP (KATO-NOGUCHI and WATADA, 1997). Plant material was ground to a fine powder, and extracted in the various buffers for 30 min, after which it was centrifuged at 30 000g. The extract was assayed as described above. Results were presented as ADH activity ($\mu M.min^{-1}.\mu g\ protein^{-1}.g\ fresh\ weight^{-1}$), and are presented in TABLE 4.1.

The most effective extraction buffer was MES A, containing the soluble PVP. However, there was still a large amount of pigment visible in the extract, which interfered with the assay. Therefore a range of PVP and Na-asc concentrations were tested to optimise the procedure further. Results are presented in TABLES 4.2 and 4.3. Form these results it was established that the most effective extraction buffer should contain 10% PVP and 15 mM of Na-asc. The final extraction buffer thus contain 100 mM MES, 2 mM DTT, 10% PVP, 15 mM Na-asc and made up to 1.25 mℓ

with deionised distilled water. Plant material (250 mg) was extracted and centrifuged as described previously. Extracts (100 µℓ) were assayed for ADH activity in both directions.

TABLE 4.1. ADH activity for the same plant material extracted in 4 different buffers. The standard errors are presented. Treatments with different bracketed letters are significantly different from one another.

Extraction buffer	ADH activity ($\mu M.min^{-1}.\mu g\ protein^{-1}.g\ fresh\ weight^{-1}$)	Standard Error
TRIS A	0.951	0.145 (a)
TRIS B	1.415	0.274 (b)
MES A	1.894	0.121 (c)
MES B	1.781	0.487 (bc)

TABLE 4.2. ADH activity for the same plant material extracted with different amounts of PVP. Standard errors are presented. Treatments with different bracketed letters are significantly different from one another.

PVP (%)	ADH activity ($\mu M\ .\ min^{-1}\ .\ \mu g\ protein^{1}\ .\ g\ fresh\ weight^{-1}$)	Standard Error
0	1.821	0.411 (a)
5	1.971	0.452 (a)
10	2.676	0.518 (b)
15	2.619	0.627 (ab)
20	2.433	0.767 (ab)

TABLE 4.3. ADH activity for the same plant material extracted in different concentrations of Na-asc. Standard errors are presented. Treatments with different bracketed letters are significantly different from one another.

Na-asc (mM)	ADH activity ($\mu M \cdot minute^{-1} \cdot \mu g\ protein^{-1} \cdot g\ fresh\ weight^{-1}$)	Standard Error
0	1.821	0.545 (a)
5	2.871	0.528 (ab)
10	3.276	0.682 (b)
15	3.919	0.625 (b)
20	2.933	0.756 (ab)

4.2.6. Pyruvate decarboxylase activity

Pyruvate decarboxylase (PDC) is the enzyme that catalyses the conversion of pyruvate to acetaldehyde. Like ADH, in the process it oxidises NADH to NAD^+, allowing its activity to be monitored using spectrophotometry. Pyruvate decarboxylase was extracted in the same buffer as the ADH (described in 4.2.5). The assay mixture contained 100 mM MES (pH 6.5), 5 mM thiamine pyrophosphate, 50 mM $MgCl_2$, 1.6 mM NADH, 13.5 units ADH and 100 µℓ plant extract. This solution was placed in a 1.5 mℓ Eppendorf tube and incubated at 30°C. The reaction was activated by the addition of 100 µℓ pyruvate, which brought the final volume of the assay mixture up to 1.25 mℓ. The PDC in the extract required thiamine pyrophosphate, a co-enzyme also known as co-carboxylase, to convert the pyruvate to acetaldehyde. The ADH present in the assay mix converted this acetaldehyde to ethanol, and indicated the amount of NADH

formed in the process. Unfortunately it was not possible to obtain a PDC standard due to its exorbitant price, and so the construction of a standard curve was not possible. Absorbance readings were recorded and expressed as absorbance.min^{-1}.µg $protein^{-1}$.g $fresh\ weight^{-1}$.

4.3. Results

The measurement of the concentration of acetaldehyde and ethanol in the micro-environment surrounding the floral head, showed that there was a small amount of ethanol present in the head space of untreated flowers (FIGURE 4.3). This may have been the result of anaerobic conditions that could have occurred while the jars were closed for gas collection. No acetaldehyde was recorded from the control flowers at any point. In treated flowers, however, high levels of both ethanol and acetaldehyde were recorded. Therefore, not only was a large amount of the applied ethanol released into the surrounding environment, but it was apparent that at least some of the ethanol was converted to acetaldehyde.

Analysis of the petal and ovary tissue for ethanol and acetaldehyde showed that in control flowers there was a slight, but not significant, accumulation of ethanol in all plant parts, under experimental conditions (FIGURE 4.4.). Acetaldehyde was barely detectable within these same control tissues. With the addition of alcohol to the holding solution however, there was a substantial increase in ethanol, and particularly acetaldehyde in the ovaries and the petals. In fact the increase in acetaldehyde concentration was over 700%.

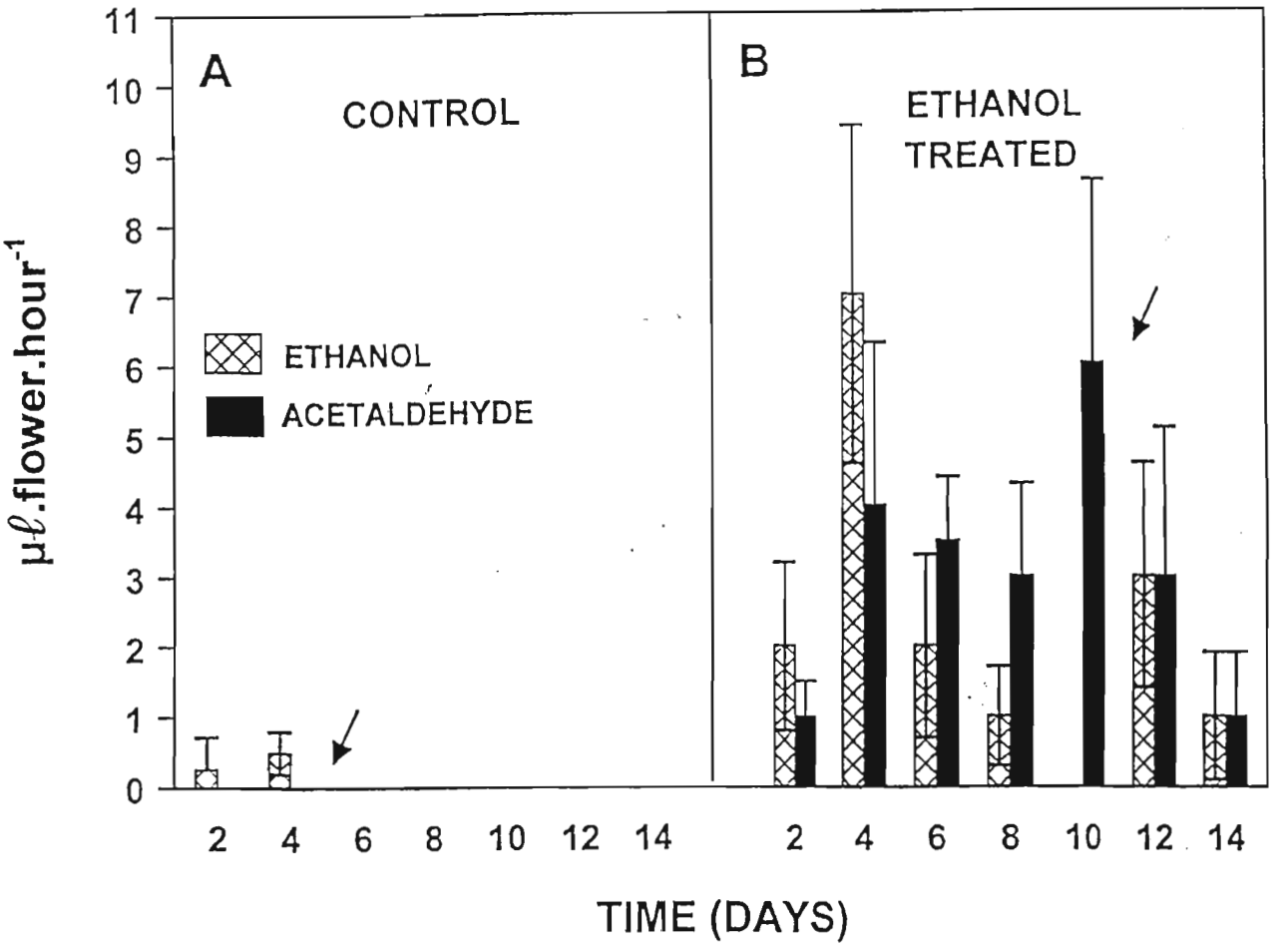


Figure 4.3. Release of ethanol and acetaldehyde by control (A) and 3% ethanol-treated (B) cut carnation flowers. Arrows represent the times S50 was reached. Bars indicate the standard errors of the results within each treatment.

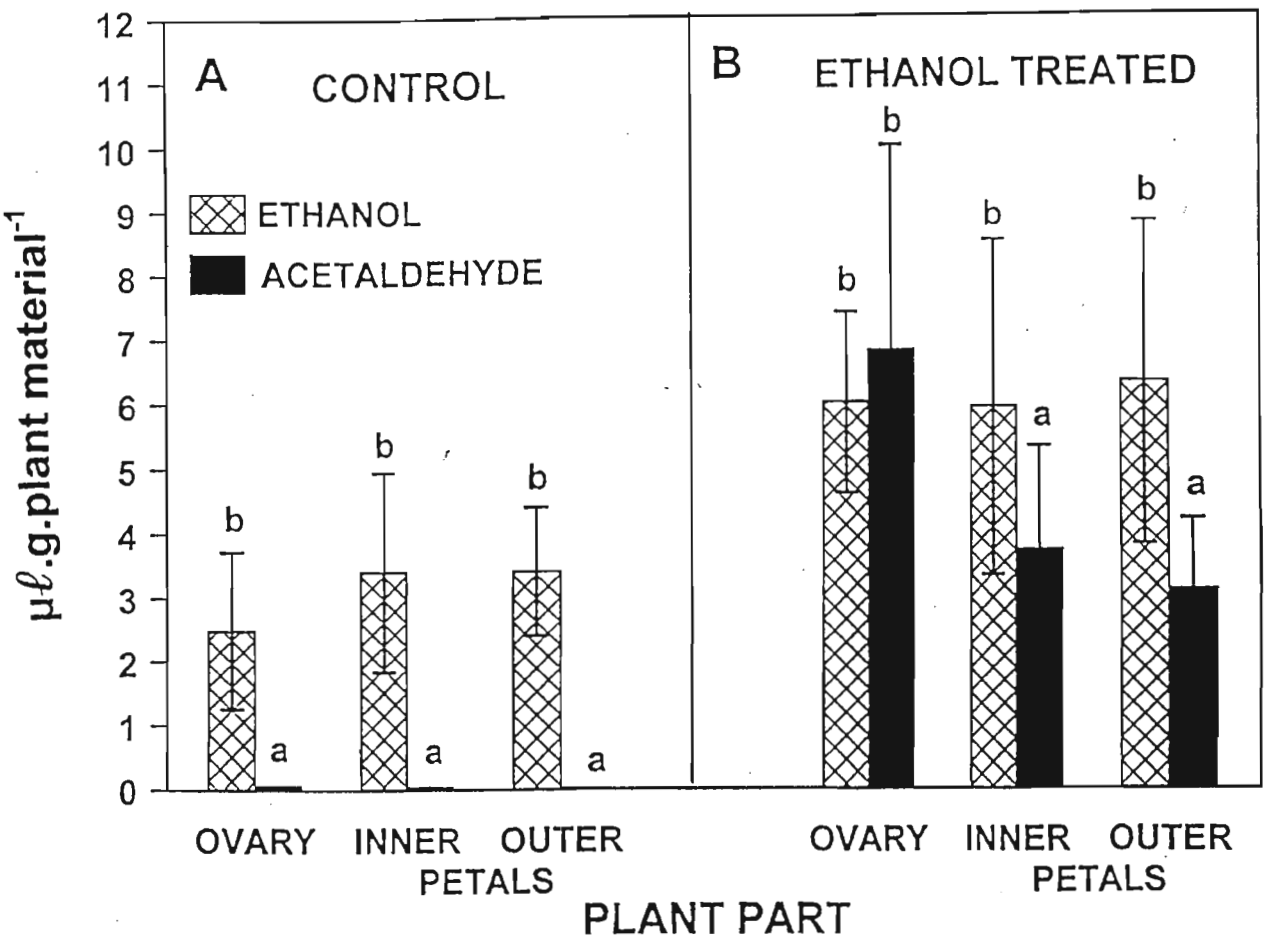


Figure 4.4. Ethanol and acetaldehyde concentrations in various parts of the flower. (A) represents levels in untreated flowers and (B) levels in 3% ethanol-treated flowers. Treatments with the same letter are not significantly different from one another. Bars indicate standard errors.

When acetaldehyde was applied to cut carnation flowers as a post-harvest treatment, an increase in vase life was recorded (FIGURE 4.5). As a pulse treatment it had immediate detrimental effects on the flowers themselves, as well as their vase life. However, treatment with a holding solution of 1 and 3% acetaldehyde increased the vase life of the flowers compared to a water control. The resultant vase life increase was not as significant as that of ethanol-treated flowers.

Application of the ADH inhibitor 4-MP, which prevents the conversion of ethanol to acetaldehyde, reduced the longevity of the flowers to a level on a par with the water control (TABLE 4.4). The ethanol-treated flowers on the other hand had a longevity increase of 200%. It therefore appeared that the formation of acetaldehyde within the tissues of ethanol-treated flowers was essential for longevity increases to occur.

Table 4.4. Longevity of flowers treated with ethanol in combination with the enzyme inhibitor 4-MP. Treatments with the same letters are not significantly different from one another.

Treatment	Longevity (S50 values)
Water	9 (a)
Ethanol	24 (b)
4-MP and ethanol	11 (a)

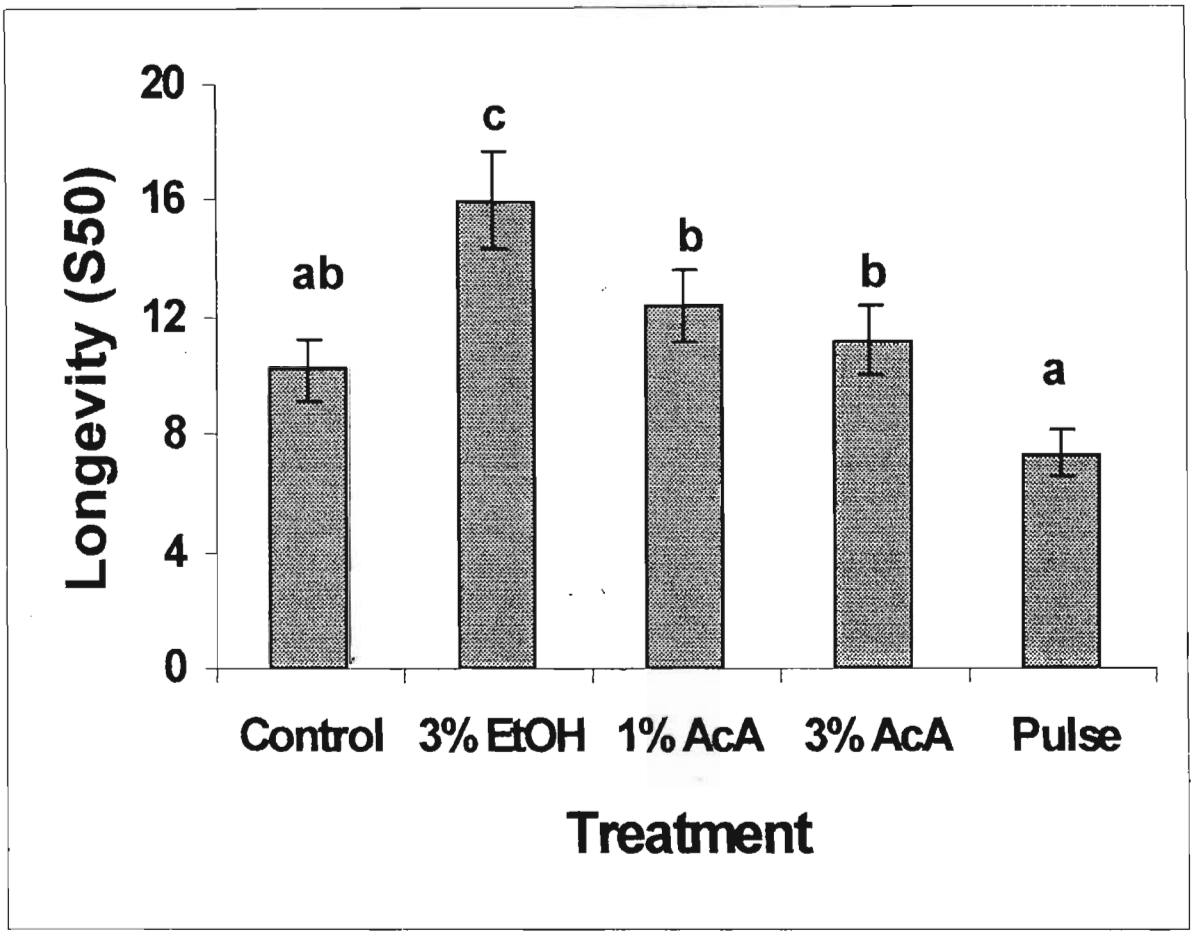


Figure 4.5. The effectiveness of acetaldehyde and ethanol (3% holding solution) treatments. AcA indicates an acetaldehyde holding solution, while pulse indicates a 15 min pulse treatment with 5% acetaldehyde. Treatments with the same letter are not significantly different from one another.

The application of the enzyme inhibitor 4-IP, which has the opposite effect as 4-MP, to cut carnation flowers resulted in a significant decrease in the vase life of these flowers when compared to flowers treated with acetaldehyde only (TABLE 4.5.). This indicated that the conversion of acetaldehyde to ethanol was also pivotal in order to achieve increased vase life. There was, however, still an increase in the longevity compared to the water control, unlike for 4-MP treated flowers.

Table 4.5. Longevity of flowers treated with acetaldehyde in combination with the enzyme inhibitor 4-IP. Treatments with the same letters are not significantly different from one another.

Treatment	Longevity (S50)
Water	7 (a)
Acetaldehyde	13 (c)
4-IP and acetaldehyde	10 (b)

The conversion of ethanol to acetaldehyde and *visa versa* was required in order to achieve any longevity increase. It was therefore necessary to determine the activity of ADH in the ethanol to acetaldehyde direction in the petals and ovaries (FIGURE 4.6.) of flowers treated with 1% acetaldehyde and 3% ethanol respectively. No reduction in ADH activity was observed compared to the water control, indicating that the enzyme had not been denatured by the ethanol. In both petal and ovary tissue treated with water and acetaldehyde there was no significant ADH activity change, while in ethanol-treated petals the activity of ADH had increased significantly.

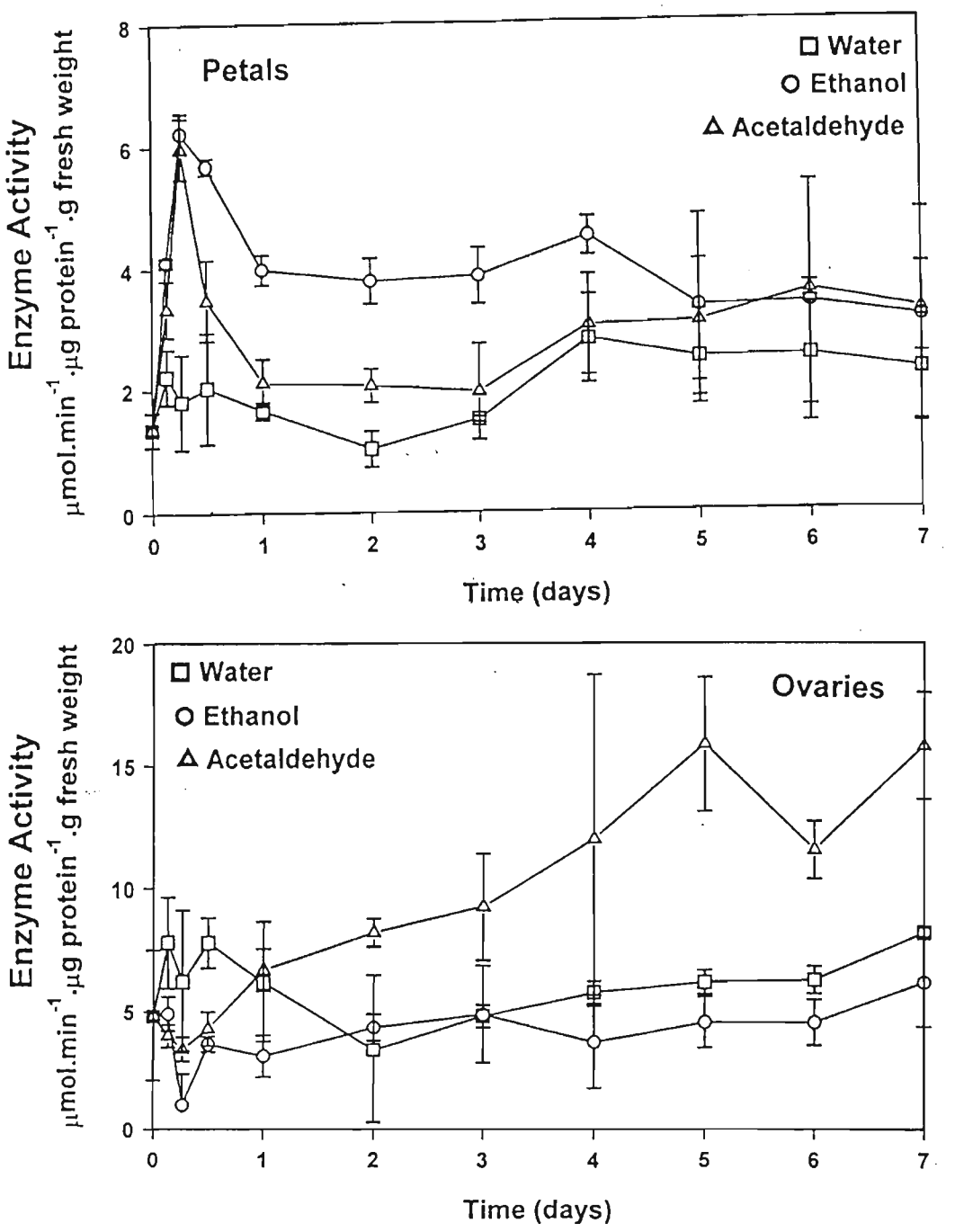


Figure 4.6. The effect of ethanol and acetaldehyde treatment on the activity of alcohol dehydrogenase (in the ethanol ➔ acetaldehyde direction). Bars show standard errors.

The conversion of acetaldehyde to ethanol was required in order to achieve longevity increases. The activity of ADH in the acetaldehyde to ethanol activity was recorded in petals and ovaries (FIGURE 4.7.). In both the petal and the ovary tissues ADH activity increased in acetaldehyde-treated flowers, while levels remained the same in ethanol-treated and control flowers. In acetaldehyde-treated petals the greatest increase was seen in the early stages of senescence, while in the ovaries, the activity increase occurred towards the end of the vase life.

Pyruvate decarboxylase (PDC) converts pyruvate to acetaldehyde within plant tissues. The effect of ethanol and acetaldehyde treatment on this enzyme was monitored in the petals and ovaries (FIGURE 4.8.). In the petals of ethanol-treated flowers there was a sudden increase in PDC activity, but this tapered off to after a few days. In acetaldehyde-treated flowers the PDC activity was initially very low, but increased until the fourth day of the experiment at which point it peaked, and decreased thereafter. Little PDC activity was recorded in the petals of the control flowers. In the ovaries, PDC activity increased throughout the experiment as a result of acetaldehyde treatment. Levels were significantly increased compared to ethanol and acetaldehyde throughout the entire experiment.

4.4. Discussion

When cut carnation flowers were treated with ethanol, it could be detected in all the organs of the flower. This indicated movement from the holding solution, via the stem, to the petals and the ovaries. Some of the applied ethanol must have been metabolised to form acetaldehyde, as it was not only detected in the micro-environment

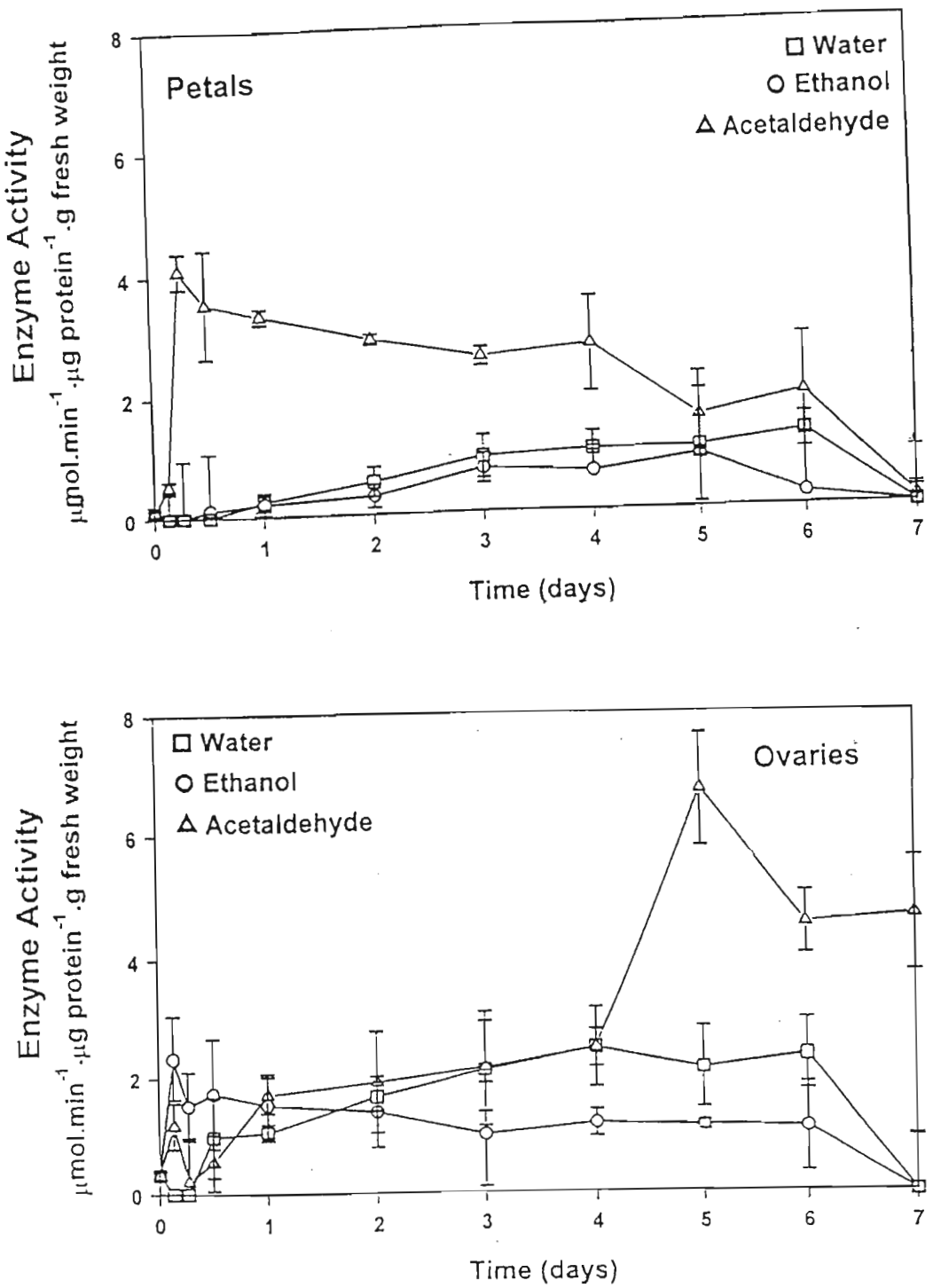


Figure 4.7. The effect of ethanol and acetaldehyde treatment on the activity of alcohol dehydrogenase (in the acetaldehyde ➔ ethanol direction). Bars show standard errors.

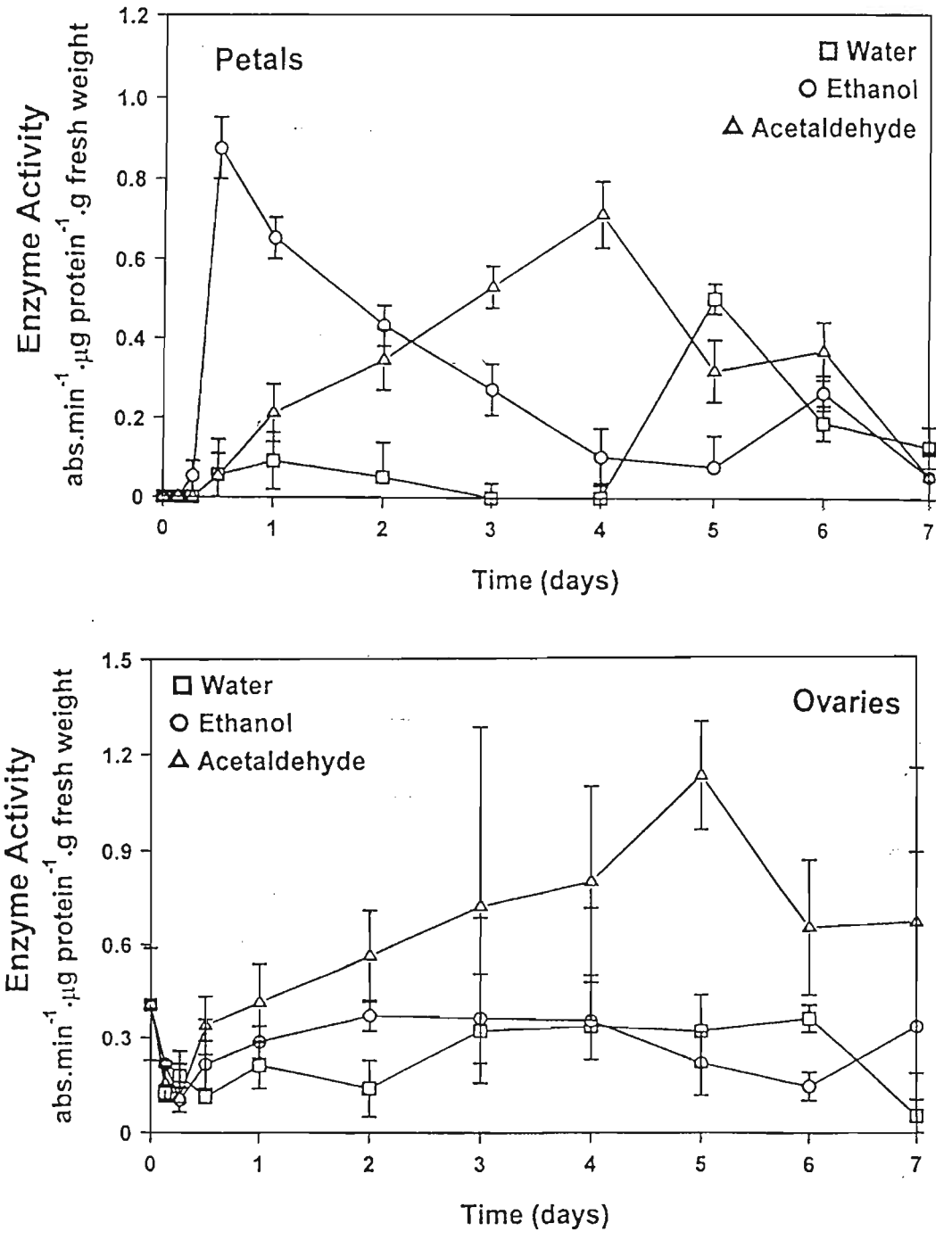


Figure 4.8. The effect of ethanol and acetaldehyde treatment on the activity of pyruvate decarboxylase. Bars show standard error.

surrounding treated flowers, but also in several of the organs, most notably the ovary. As in the case of the inhibition of ripening seen in ethanol-treated tomatoes (BEAULIEU, PEISER and SALTVEIT 1997), it was easy to conclude that this metabolised acetaldehyde was the causal agent of ethanol-induced increases in flower longevity.

There were two main reasons for reaching this conclusion. Firstly, very high levels of acetaldehyde were recorded in the ovaries of ethanol-treated flowers. The ovary is the organ most significantly affected by ethanol treatment. Under normal senescence circumstances the ovary acts as a sink for carbohydrates within the system, gaining in dry weight as senescence continues. This sink activity of the ovary causes the petals to act as a source. As they loose carbohydrates, the petals begin to roll inwards, and it is this loss of diameter and the resultant decrease in aesthetic appeal, that leads to the end of the vase life of these flowers (DIMALLA and VAN STADEN, 1980). However, when flowers were held in 2% ethanol, they did not gain weight or increase in diameter (HEINS and BLAKELY, 1980). If the ovary is no longer acting as a carbohydrate sink, it seems likely that the petals are no longer acting as a source. This at least partially explains the increased vase life of ethanol-treated flowers. If the ovary is the organ of the cut carnation flower most effected by ethanol treatment, it is also the organ in which the greatest concentration of acetaldehyde was recorded as a result of ethanol treatment. Levels of the compound increase some 700%, and in fact there is more acetaldehyde present in ethanol-treated ovaries than ethanol. This suggests that the failure of ovaries of treated flowers to gain weight and increase in diameter may be entirely as a result of acetaldehyde formation, or possibly the presence of both ethanol and acetaldehyde within the tissue, and their effect of cell

structure. With such large acetaldehyde levels being recorded it seems unlikely that only ethanol is responsible for the impact of ethanol treatment on the ovary.

The other reason for suggesting that acetaldehyde is essential for the ethanol-induced longevity increases seen in cut carnation flowers, is prompted by the fact that no longevity increase occurred when acetaldehyde was not allowed to form within the system. When 4-MP was applied to the flowers in combination with ethanol, senescence was accelerated compared to normal treated flowers. This implied that acetaldehyde was at least partially responsible for whatever physiological changes occurred within the system and resulted in increased longevity. Examples of physiological responses to ethanol treatment were the inhibition of ethylene synthesis (HEINS, 1980), ethylene action (WU, ZACHARIAS, SALTVEIT and REID, 1992) and a reduction in the climacteric respiration rate (HEINS and BLAKELY, 1980).

When acetaldehyde was applied directly to the flowers as a post-harvest treatment there was a significant increase in the vase life of the flowers. This indicated that not only was acetaldehyde involved in causing the longevity increase seen when ethanol was used as a post-harvest treatment, but there was also the potential for acetaldehyde itself to be used as a post-harvest treatment.

Acetaldehyde does not act alone to increase flower longevity. When 4-IP was applied to the system, the conversion of acetaldehyde to ethanol was inhibited, and there was a decrease in the resultant vase life compared to flowers treated with acetaldehyde only. Therefore the presence of ethanol was also required within the system in order to have the maximum longevity increase. However, the flowers treated

with 4-IP had a significantly longer vase life than the control flowers. This indicated that even when ethanol was excluded from the system, acetaldehyde still promoted the vase life of the flowers. While flowers treated with ethanol did experience a significant increase in vase life, no longevity increase occurred if acetaldehyde was not allowed to form within the system.

When ethanol was applied to cut carnation flowers a longevity increase was recorded only if some of the absorbed ethanol was converted to acetaldehyde. The enzyme required for this conversion is ADH, in the ethanol to acetaldehyde direction. In ethanol-treated flowers an increase in the activity of this enzyme was observed in both the petals and ovaries of the flowers. Acetaldehyde also increased flower longevity only if the conversion to ethanol was allowed, and ADH activity in the acetaldehyde to ethanol activity was found to increase in both petals and ovaries. The level of the enzyme in opposite orientation to the applied treatment (i.e. the activity of ADH in the acetaldehyde to ethanol direction in ethanol-treated flowers) was also increased compared to the water control. Pyruvate decarboxylase converts pyruvate to acetaldehyde. The activity of this enzyme was also increased in the ovary and petal tissue of flowers treated with ethanol and acetaldehyde. Together with the results of the 4-MP and 4-IP experiments, this indicated that a large degree of inter-conversion between ethanol and acetaldehyde, and possibly even pyruvate, occurred during treatment with either compound.

CHAPTER 5

THE POTENTIAL USE OF ACETALDEHYDE AS A POST-HARVEST TREATMENT

5.1. Introduction

The application of ethanol to cut-carnation flowers resulted in increased longevity, decreased ethylene production and decreased sensitivity to exogenously applied ethylene (HEINS, 1980; HEINS and BLAKELY, 1980; WU, ZACHARIAS, SALTVEIT and REID, 1992; Chapter 2). Ethanol also prevents the ripening of certain fruits (KELLY and SALTVEIT, 1988; SALTVEIT and MENCARELLI, 1988; MENCARELLI, SAVARESE and SALTVEIT, 1991; PESIS and MARINANSKY, 1993). In both fruits (BEAULIEU, PEISER and SALTVEIT, 1997) and flowers (Chapter 4) the formation of acetaldehyde within treated tissues was necessary, if not responsible, for the longevity increases seen after ethanol application. Direct acetaldehyde application has been used successfully to prevent ripening in tomatoes, mangoes, kiwifruit and grapes (KELLY and SALTVEIT, 1988; MENCARELLI, SAVARENSE and SALTVEIT, 1991; PESIS and MARINANSKY, 1992; PESIS and MARINANSKY, 1993; BEAULIEU, PEISER and SALTVEIT, 1997).

The aim of the experiments performed in this study was to determine whether a direct application of acetaldehyde would increase the longevity of cut carnation

flowers, and whether acetaldehyde could be a commercially viable post-harvest treatment.

5.2. Materials and methods

5.2.1. Acetaldehyde solutions

Acetaldehyde forms a dimer at room temperature. It was therefore essential to freshly distill all acetaldehyde that was to be applied to flowers. As acetaldehyde is a noxious chemical that can lead to respiratory problems, it was essential to prevent as much acetaldehyde evaporation as possible. Pouring was done in a fume hood, and tin foil caps were placed firmly over the holding vessels. As with ethanol application, holding solutions were used and refreshed every two days.

5.2.2. Other materials and methods

All other methods and materials, for plant material, holding the flowers, pulse treatments, S50 and S100 values, statistical analysis, ethylene determination and exogenous ethylene were the same as those used in Chapter 2. A 3% ethanol control, as well as a water control was used for all longevity trials.

5.3. Results

Nine concentrations of acetaldehyde were applied to cut carnation flowers, ranging from 0% to 8%, and the resultant longevity of the flowers was compared to both a water control, and a 3% ethanol solution. Solution concentrations were of a lower concentration than those tested for ethanol, as it was indicated in the literature

that acetaldehyde should be applied in lower concentrations than ethanol in the same system i.e. tomatoes (SALTVEIT, 1989). Longevity results of five repetitions of the experiment, as well as the mean longevity increase, are shown in TABLE 5.1.

Table 5.1. The longevity of cut carnation flowers (S50) treated with various concentrations of acetaldehyde. Std Dev = standard deviation. Eth = 3% ethanol treatment. Treatments with the same letters are not significantly different from one another.

Repetition of the experiment	Acetaldehyde treatment (%)									
	0	**0.1**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**8**	**Eth**
1	7	6	5	6	12	13	12	12	12	16
2	10	9	8	10	14	15	16	14	13	18
3	9	7	8	8	12	13	12	12	11	17
4	12	11	10	11	18	16	18	17	16	21
5	6	7	6	7	10	10	12	11	10	14
Mean	**8.8a**	**8.0a**	**7.4a**	**8.4a**	**13.2b**	**13.4b**	**14b**	**13.2b**	**12.4b**	**17.2b**
Std Dev	**±2.4**	**±2.0**	**±1.95**	**±2.07**	**±3.03**	**±2.3**	**±2.83**	**±2.39**	**±2.3**	**±2.59**

All concentrations above 1% acetaldehyde increased the longevity of the flowers when compared to the water control. No concentration between 1.5% and 4% was significantly more effective than any other, resulting in similar appearance of the flowers (PLATE 5.1). The longevity of flowers treated with 3% ethanol was greater than the longevity of any of the acetaldehyde treatments. A low concentration of acetaldehyde (1.5%) was chosen as the standard holding solution for further experiments. A limitation of acetaldehyde as a post-harvest treatment

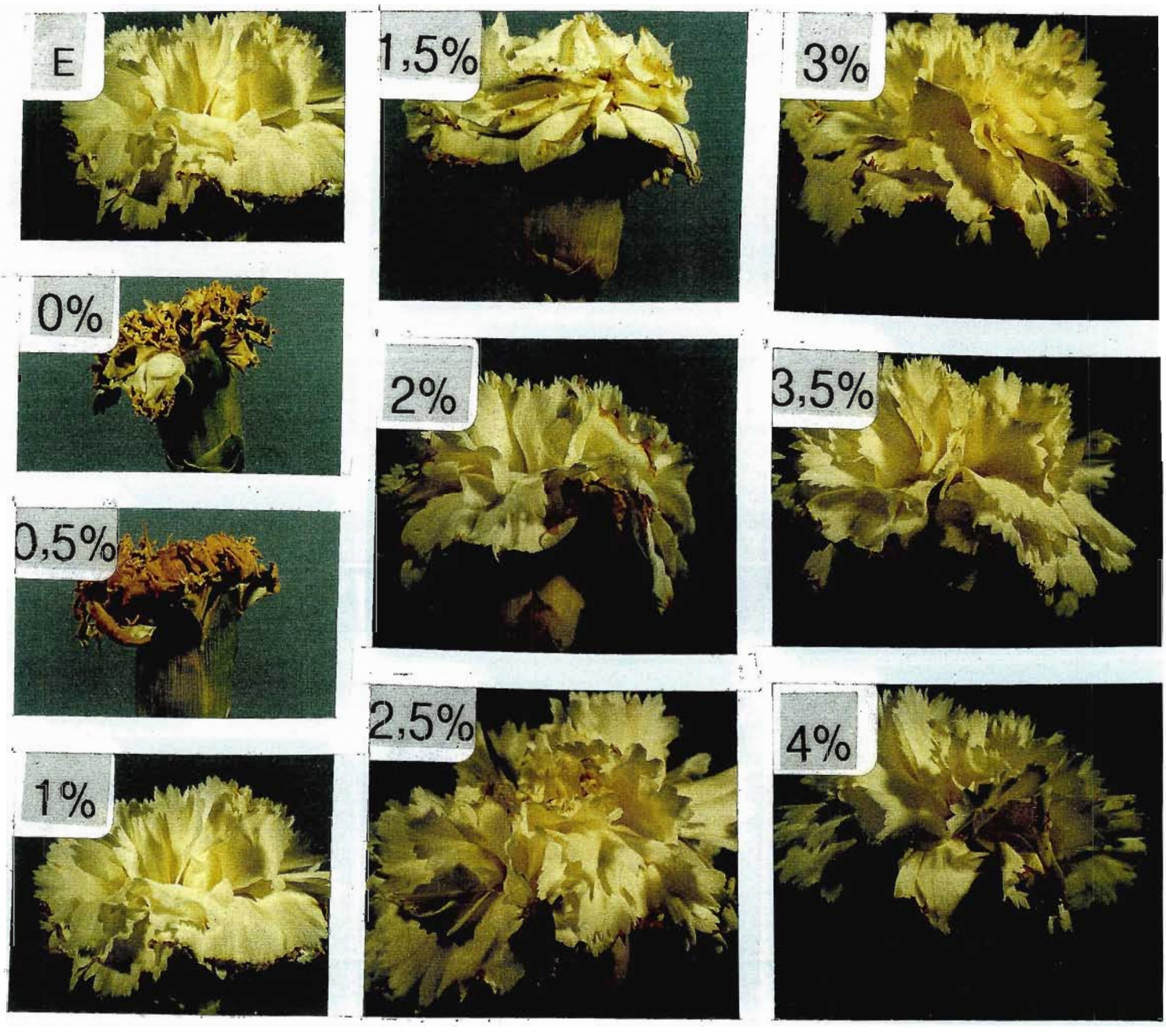


Plate 5.1. The effect of various concentrations of acetaldehyde on the physical appearance of cut carnation flowers (Day 7). Text boxes indicate the concentration of acetaldehyde used. E = 3% ethanol treatment.

was immediately encountered when it was determined that, like ethanol, the same concentration of acetaldehyde was not the most effective when the experiments were repeated.

The flowers treated with 0.5% acetaldehyde were 50% senesced slightly earlier than those exposed to no acetaldehyde at all. Acetaldehyde has been shown to promote fruit softening in pears (JANES and FRENKEL, 1978), and stimulate the ripening of blueberries, pears and Japanese persimmons (IMATURA, 1986; JANES and FRENKEL, 1978; PAZ, JANES, PREVOST and FRENKEL 1981). This appeared to be the case in carnation flowers as well. There was not a significant promotion of senescence in these flowers, but it is possible that if lower concentrations of acetaldehyde were applied, a greater promotion of senescence may have occurred, as the ability of acetaldehyde to both promote and inhibit ripening appears to be concentration related.

As for ethanol, the next factor tested was whether acetaldehyde could be applied as a pulse treatment (FIGURE 5.1). High concentration pulses, containing 5, 10 and 30% acetaldehyde, were applied to flowers for 4 and 24 h. The longevity was compared with flowers held in a 1.5% acetaldehyde holding solution. The longevity of flowers pulsed with acetaldehyde for 4 h, regardless of the concentration, was not significantly different from the water control. All of the 24 h pulse treatments resulted in a significant decrease in the vase life compared to both the 1.5% acetaldehyde holding solution and the water control.

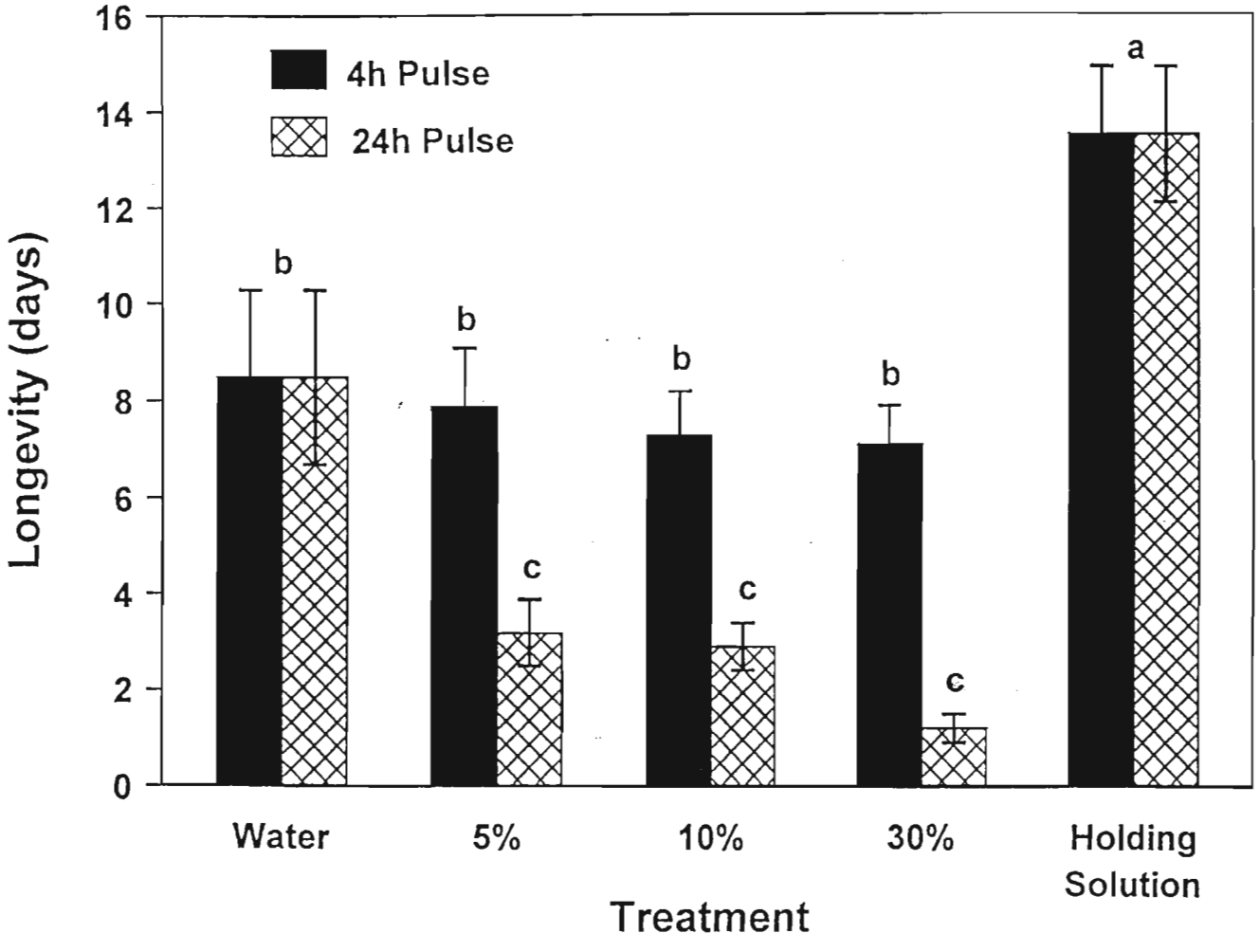


Figure 5.1. The effectiveness of various acetaldehyde concentrations on carnation flower longevity when applied as 4 and 24 h pulses. Bars indicate the standard error for each treatment. Treatments with the same letters are not significantly different from one another.

Like ethanol, it appeared that acetaldehyde could not be applied as a pulse treatment. The effect of application time on the resultant longevity of treated flowers was determined (FIGURE 5.2.). Apart from an unusual result for the one day application, a linear response was observed, indicating that the longer the acetaldehyde application, the greater the resultant increases in vase life. Compared to the water control, the increase in vase life of treated flowers was not significant unless applied for six days or longer. The greatest increase in vase life was achieved with the continuous acetaldehyde treatment, indicating that like ethanol, acetaldehyde needed to be applied continuously.

Various concentrations of acetaldehyde were applied to cut carnation flowers and the resultant ethylene production was recorded (FIGURE 5.3.). At concentrations of 1% acetaldehyde and higher no ethylene was produced at all, the climacteric ethylene production was completely inhibited. At concentrations below 1% however, some ethylene was recorded, although at levels significantly lower than in untreated flowers. While acetaldehyde, like ethanol, inhibited the production of ethylene, it appeared that there was some kind of concentration threshold that must be reached before this effect could be seen.

Exogenously applied ethylene results in climacteric ethylene production by control flowers, and premature senescence. If a post-harvest treatment prevents this senescence it must be active against the action of ethylene. Ethylene was applied to flowers in the form of Ethrel ® in conjunction with 1.5% acetaldehyde (FIGURE 5.4.). Acetaldehyde resulted in no increase in longevity compared to the water control, indicating that it does not prevent that action of ethylene. In fact, on a few

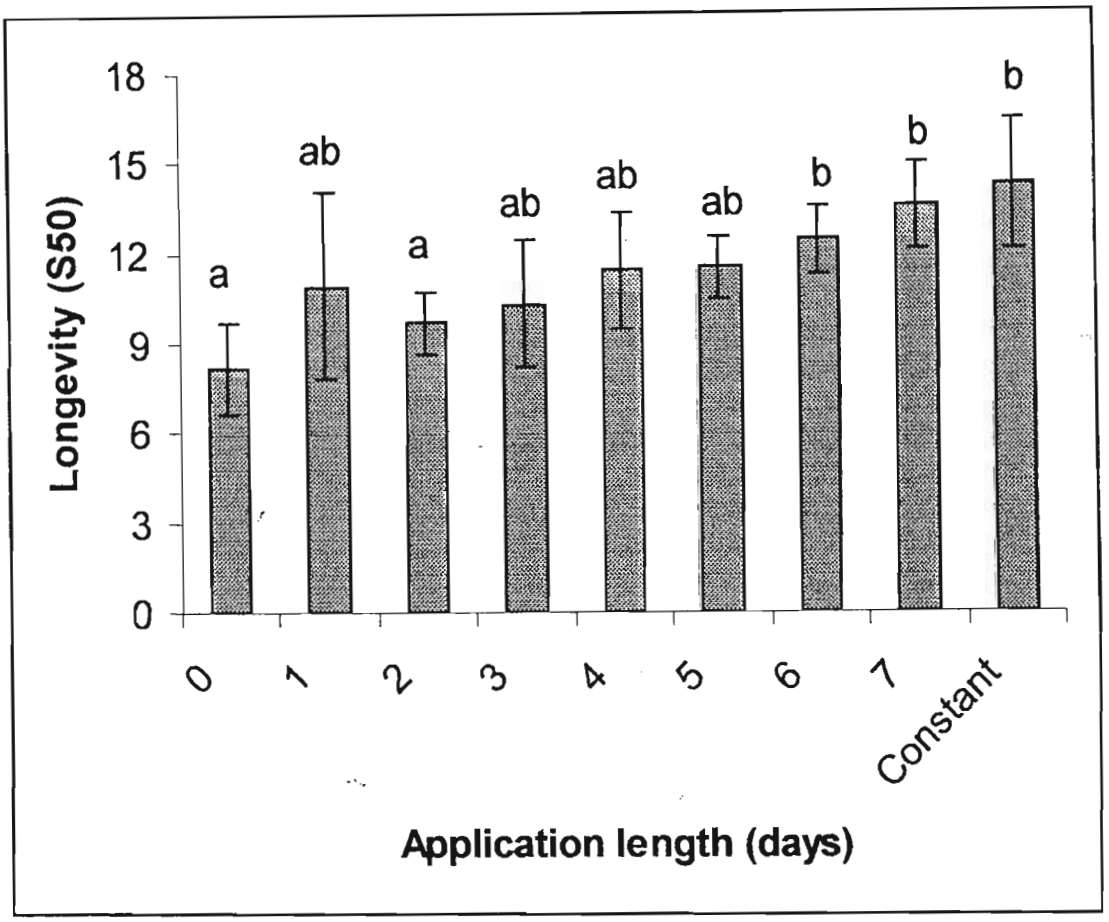


Figure 5.2. The effect of the duration of acetaldehyde application on the resultant vase life of cut carnation flowers. Bars indicate standard errors within the treatments. Treatments with the same letter are not significantly different from one another.

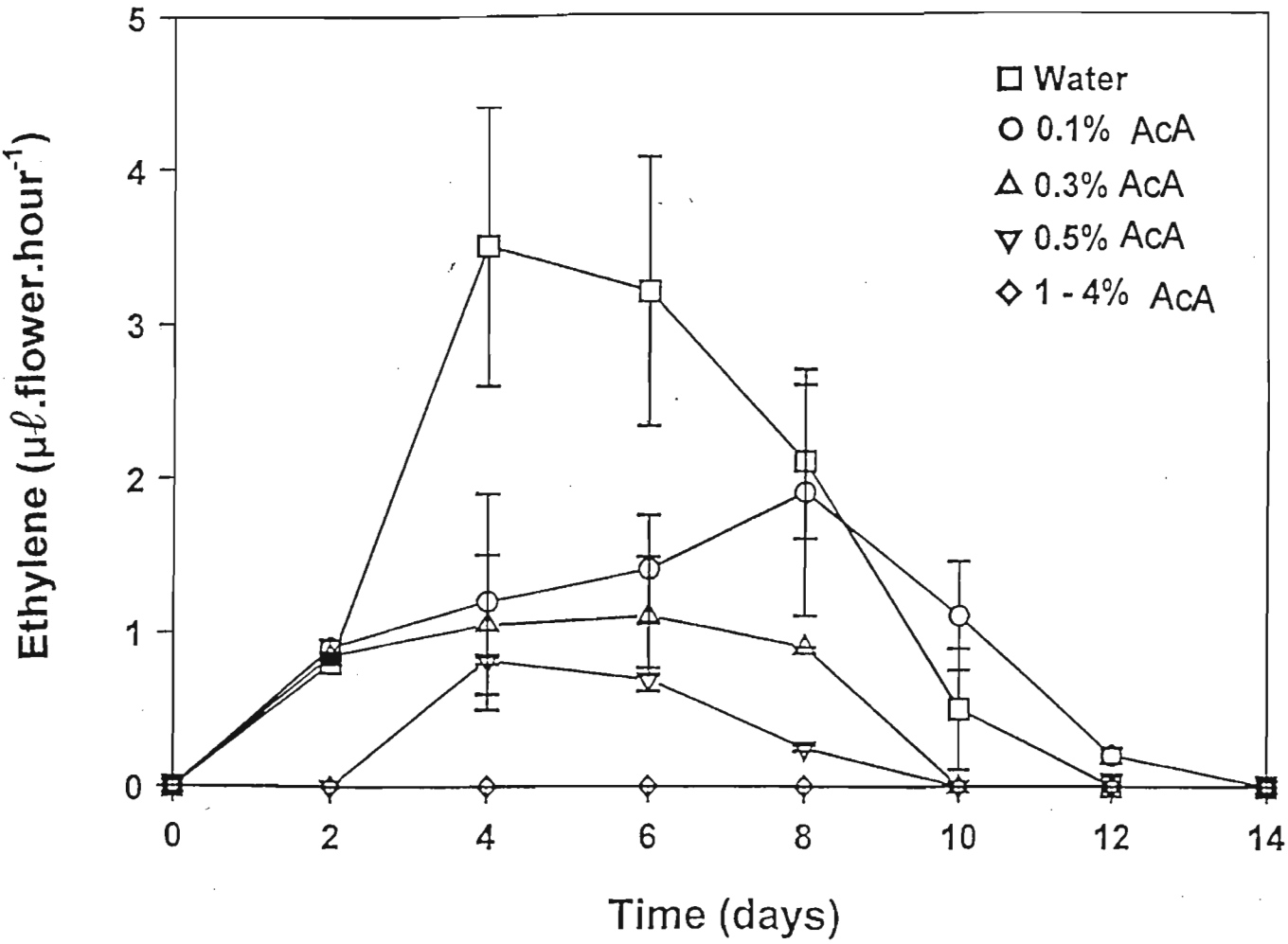


Figure 5.3. The effect of various acetaldehyde concentrations on ethylene production in carnation flowers. Bars indicate the standard errors for each of the treatments.

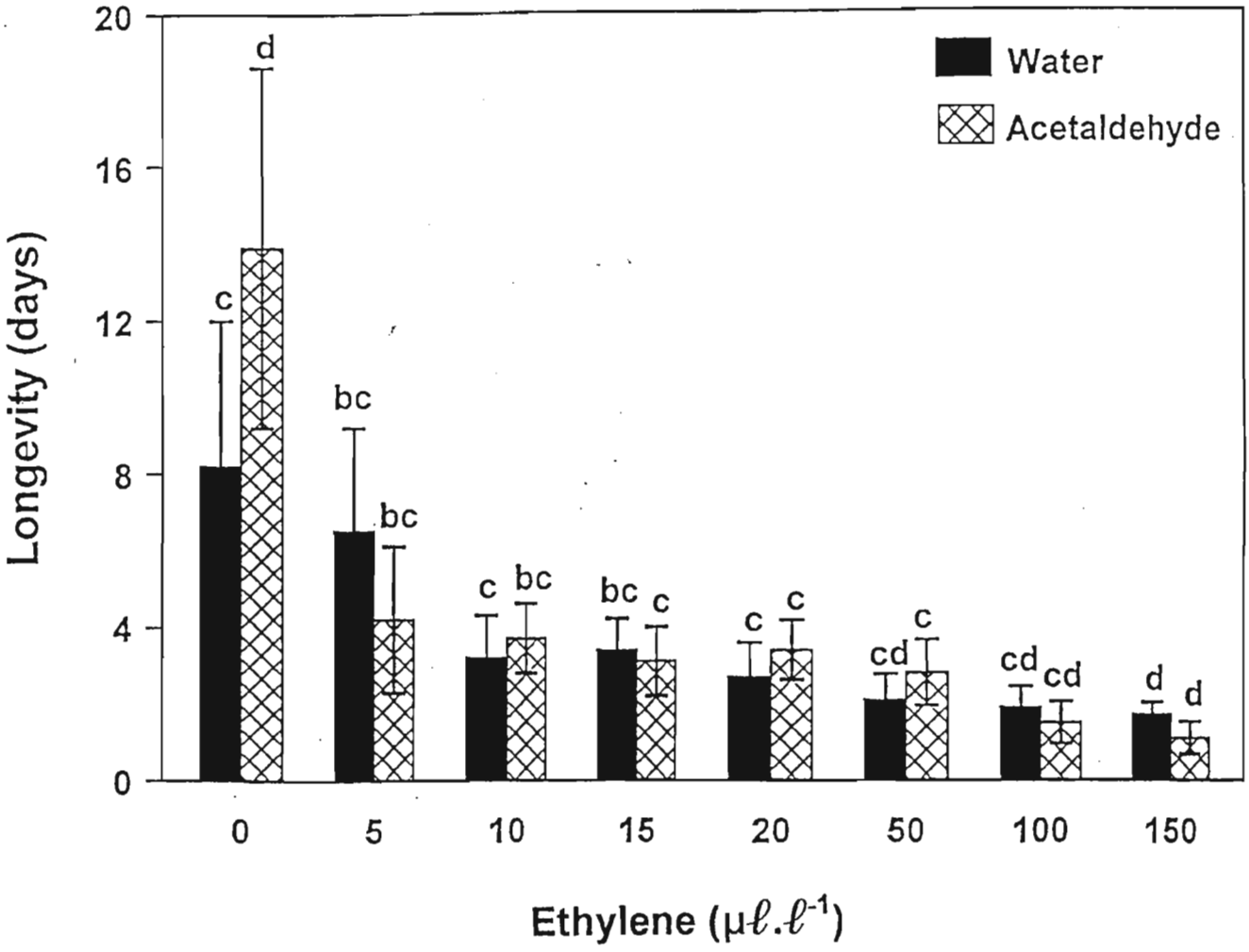


Figure 5.4. The effect of exogenously applied ethylene and 1.5% acetaldehyde on cut carnation flower longevity. Bars indicate standard error for each treatment. Treatments with the same letter are not significantly different from one another.

occasions, the combination of Ethrel (5, 15, 100 and 150 $\mu\ell.\ell^{-1}$) and acetaldehyde resulted in reduced vase life compared to flowers treated with water. This may be linked to the production of ethylene by some concentrations of acetaldehyde-treated flowers and the promotion of senescence by low concentrations of acetaldehyde.

If the onset of acetaldehyde application was delayed, the resultant vase life of the flowers was reduced (FIGURE 5.5.). As in ethanol treatment, there was a linear relationship between the delay in acetaldehyde treatment, and the subsequent vase life of the flowers. Climacteric ethylene production for this group of flowers occurred on day 5. When treatment began before day 5 there was still an increase in the longevity compared to the water control. However, when treatment began after day 5, after climacteric ethylene production had occurred, there was no difference between the vase life of flowers treated with acetaldehyde and control flowers.

Acetaldehyde exhibited a major effect when the stem length of cut carnation flowers was varied (FIGURE 5.6). For ethanol treatment, the longer the stem the greater the longevity increase. For 1.5% acetaldehyde-treated flowers an opposite relationship was observed. The greatest vase life was recorded in the flowers with the shortest stems. The massive reduction in the vase life of the flowers with the longest stems was due to stem "toppling", which occurs when the stem is weakened by treatment, and can no longer support the floral head. Stem "toppling" effectively ended the vase life of the flowers, regardless of the state of the petals (PLATE 5.2).

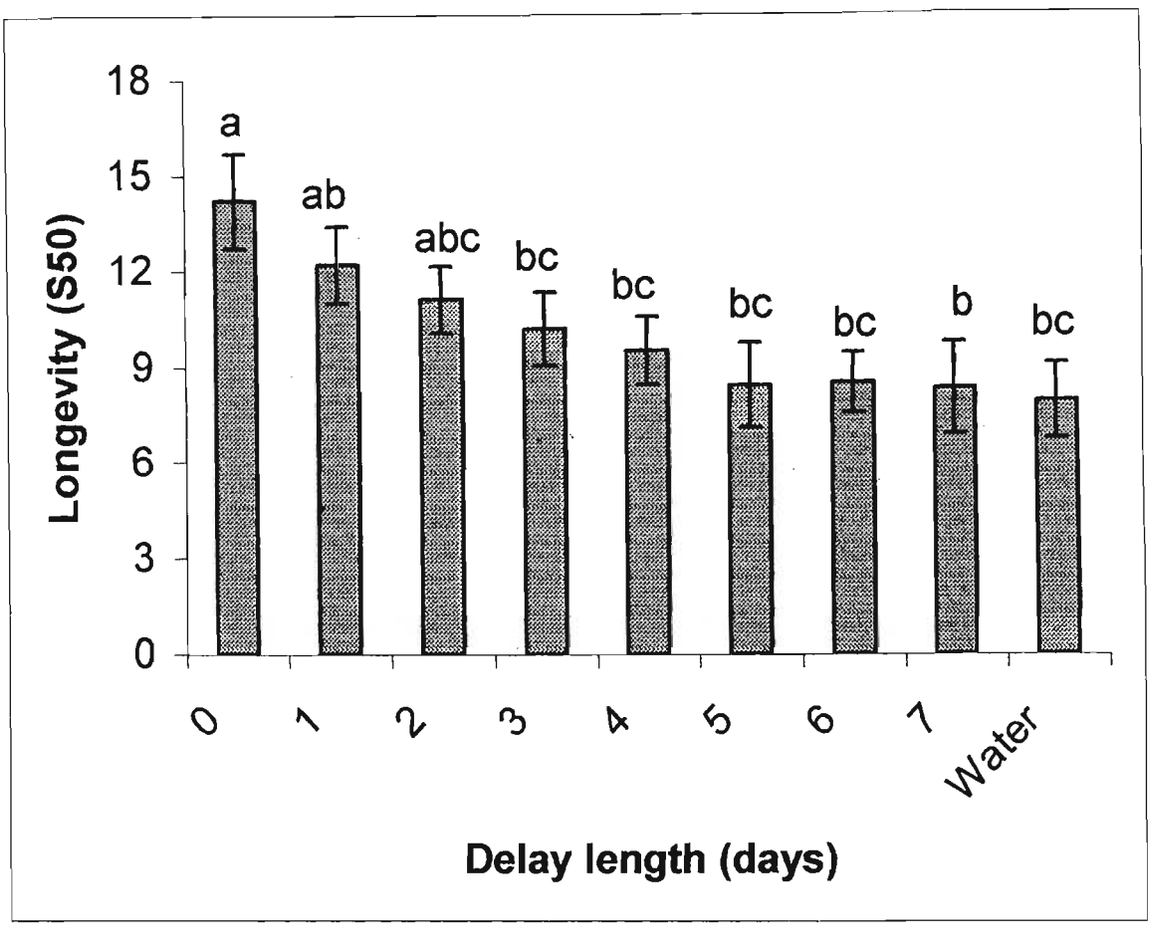


Figure 5.5. The effect of a delay in acetaldehyde application on the vase life of cut carnation flowers. Water represents the water control. Bars indicate standard error. Similar letters indicate treatments that are not significantly different from one another.

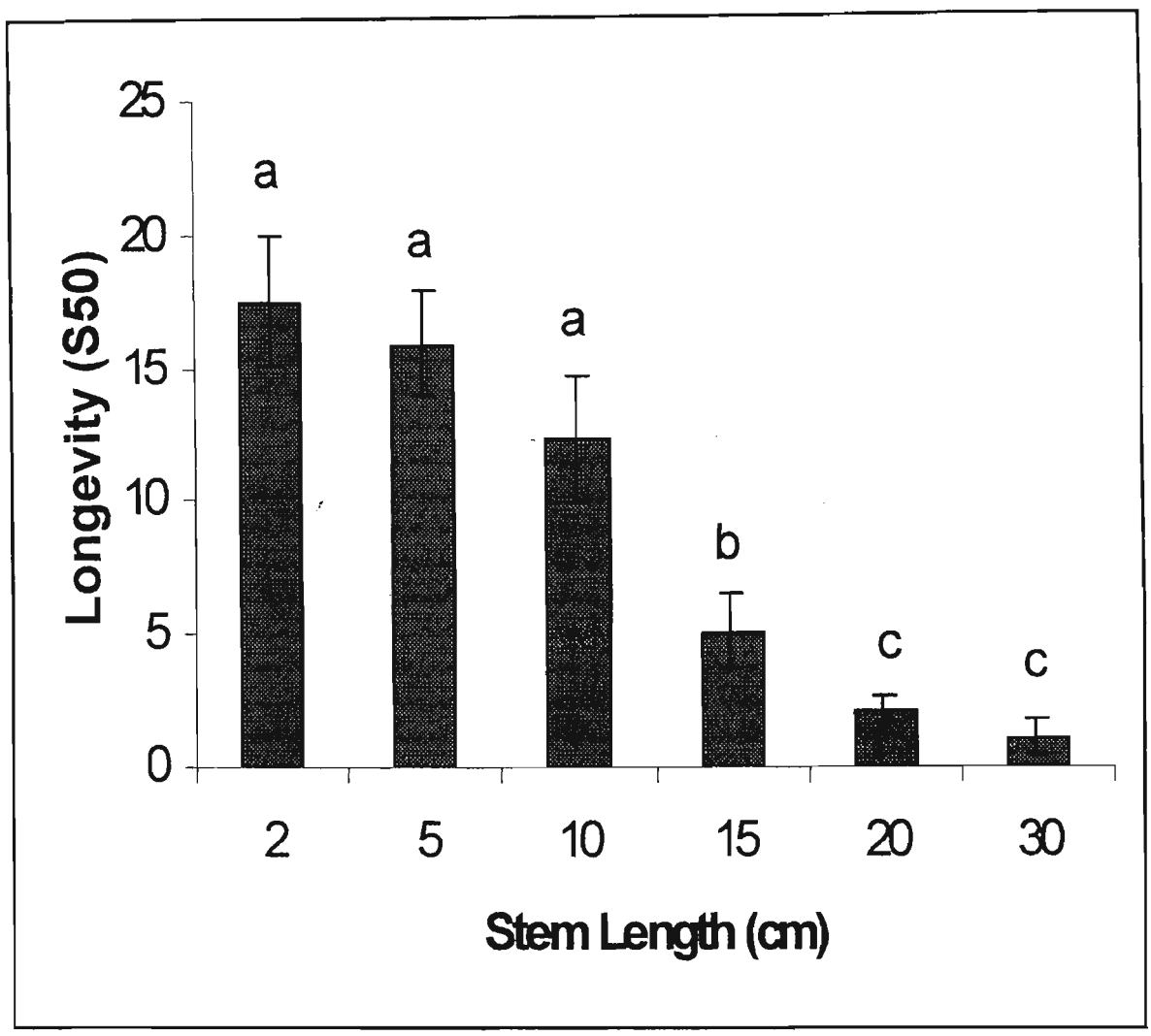


Figure 5.6. The effect of 1.5% acetaldehyde on cut carnation flower longevity when the length of the flower stems was varied. Bars indicate standard error within the treatments. Treatments with the same letter are not significantly different from one another.

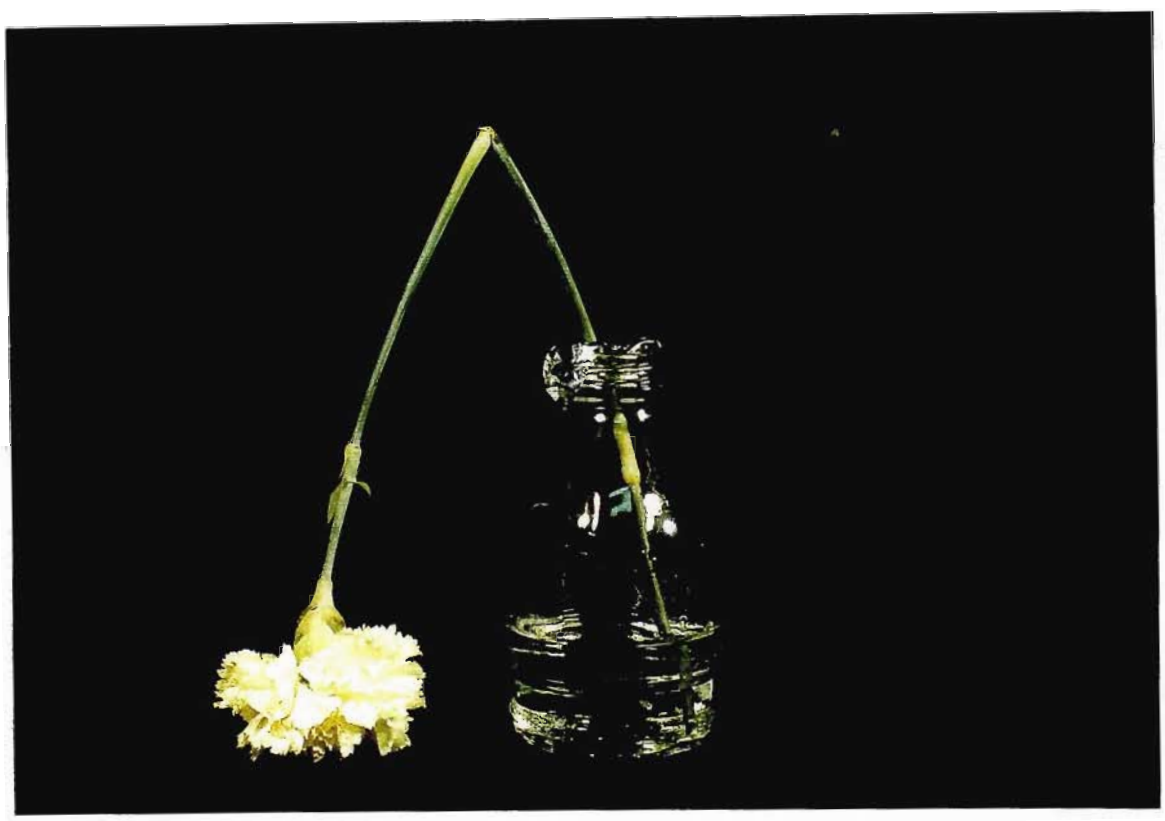


Plate 5.2. An acetaldehyde-treated flower displaying the phenomenon of stem toppling on the second day of experimentation.

5.4. Discussion

The correlative process of senescence in cut carnation flowers is mainly due to the production of the plant hormone, ethylene. Acetaldehyde-treated cut carnation flowers no longer produce ethylene (the mechanism for this inhibition of ethylene formation will be discussed further in Chapter 6). This confirms that the response seen with acetaldehyde treatment in many fruits is also seen in cut carnation flowers (PESIS and MARINANSKY, 1992; PESIS and MARINANSKY, 1993; BURDON, DORI, LOMANIEC, MARIANSKY and PESIS, 1994; BURDON, DORI, MARINANSKY and PESIS, 1996; BEAULIEU and SALTVEIT, 1997). If there is no ethylene formation, the symptoms of ethylene-induced senescence, such as petal "sleepiness" and ovary development will not occur. Instead the floral head will remain unaffected by the severing of the flower from the parent, thereby extending the vase life of the flowers. On average, acetaldehyde treatment at concentrations above 1% extended the vase life of carnation flowers 150% compared to the water control. However, this acetaldehyde could not be applied as a pulse treatment or for a short period of time only. It was necessary for the acetaldehyde solution to be present throughout the vase life of the flowers, otherwise shortened vase life ensued. Any delay in acetaldehyde treatment shortened the vase life of the flowers, and if climacteric ethylene production had already begun there was no increase in the vase life at all.

As with ethanol treatment, in order for acetaldehyde to be an efficient post-harvest treatment, it must delay or arrest the senescence process in cut carnation flowers. Unfortunately, although the discovery that acetaldehyde increases the vase life of cut carnation flowers is novel, acetaldehyde, like ethanol, is not a suitable

commercial post-harvest treatment. Even though it prevented the formation of ethylene, acetaldehyde proved to be ineffective at preventing the action of exogenously applied ethylene. This will result in large-scale transportation problems. That a pulse treatment is not effective is also a problem for a potential commercial post-harvest treatment. In addition acetaldehyde treatment resulted in "toppling" and weakening of the stem almost immediately after the onset of treatment, thereby ending the vase life of the flowers early. The only flowers in which no stem bending was recorded were those with stems of 10 cm or shorter. The longer the stem, the more severe the bending. Stem bending may have occurred due to extensive damage to the cells and tissues of the stem, as acetaldehyde is a phytotoxic compound. Any damage to the cell wall or membrane would result in the stem weakening. The longer the stem, the greater the weight of the floral head, explaining why the bending was more severe in long-stemmed flowers. Cell damage by acetaldehyde, as well as phytotoxicity will be discussed in detail in Chapter 6.

There were a large number of similarities between the response of flowers to acetaldehyde treatment and ethanol treatment. The inhibition of ethylene formation, the ineffectiveness of a pulse treatment, the need for a constant holding solution, the effect of a delay in the onset of treatment and the lack of inhibition of ethylene action were common to both treatments. The only major difference between the two treatments was that acetaldehyde treatment resulted in stem bending. This was not recorded for ethanol treatment, though. It was observed in flowers treated with other alcohols however (WU, ZACHARIAS, SALTVEIT and REID, 1992). The similarity in results implies that the two post-harvest treatments were very closely tied to one another. This can be explained by the fact that ethanol and acetaldehyde are

interchangeable with each other during the treatment process, as was suggested in Chapter 4. If both ethanol and acetaldehyde were involved in the responses, it would explain why they were almost exactly the same. This concept will be examined more closely in Chapter 7.

A possible promotion of senescence by acetaldehyde treatment was recorded during the longevity trials. Although never significantly lower, the actual vase life of flowers treated with 0.1, 0.3 and 0.5% acetaldehyde was always shorter than that of the water control. Acetaldehyde has been reported to promote fruit ripening. In pears an increase in the internal level of acetaldehyde resulted in increased ripening (JANES and FRENKEL, 1978), and exogenous applications of acetaldehyde promote the ripening of blueberries, pears and Japanese persimmons (JANES and FRENKEL, 1978; PAZ, JANES, PREVORST and FRENKEL, 1981; IMATURA, 1986). An explanation of the dual action of acetaldehyde, (i.e. the promotion and inhibition of senescence/ripening) may lie in its concentration. Only very low concentrations of acetaldehyde (0.03 – 0.17%) were found to promote pear ripening. When concentrations were increased to 0.3% there was no difference between the ripening time of these fruits and the control (JANES and FRENKEL, 1978). Similarly low levels were used to promote grape ripening (PESIS and FRENKEL, 1989) and respiration in tomatoes, blueberries and pears (PAZ, JANES, PREVORST and FRENKEL, 1981). When acetaldehyde was applied to inhibit fruit ripening and flower senescence the concentrations required were significantly higher (KELLY and SALTVEIT, 1988; SALTVEIT and MENCARELLI, 1988; PESIS and MARINANSKY, 1993). This concentration-dependant promotion of fruit ripening appears to be unrelated to ethylene production. Possible mechanisms for the promotion include

the utilisation or metabolism of the compound, which has been shown to affect the Krebs cycle, electron transfer and lipid metabolism (CEDERBAUM, LIEBER and RUBIN; 1974; CEDERBAUM, LIBER and RUBIN, 1975; JANES and FRENKEL, 1978).

CHAPTER 6

THE PHYSIOLOGICAL RESPONSE OF CUT CARNATION FLOWERS TO ACETALDEHYDE AS A POST-HARVEST TREATMENT

6.1. Introduction

Fruit treated with acetaldehyde exhibit a number of physiological responses. In blueberries and tomato fruit, acetaldehyde application increased the concentration of reducing sugars (PAZ, JANES, PREVORST and FRENKEL, 1981). Acetaldehyde treatment also stimulated the respiration of apple, blueberry, orange, grape and strawberry fruits, as well as potatoes (FIDLER 1968; JANES, CHIN and FRENKEL, 1978; PESIS and MARINANSKY, 1992). It is well documented that the production of ethylene is dramatically reduced in fruit treated with acetaldehyde. Ethylene inhibition by acetaldehyde has been reported in peaches (LURIE and PESIS, 1992), kiwifruit (MENCARELLI, SAVARESE and SALTVEIT, 1991), grapes (PESIS and MARINANSKY, 1992), bananas (HEWAGE, WAINWRIGHT and LUO, 1995), mangoes (BURDON, DORI, LOMANIEC, MARINANSKY and PESIS, 1996) and tomatoes (BEAULIEU and SALTVEIT, 1997).

The inhibition of fruit ripening of tomatoes (BEAULIEU, PEISER and SALTVEIT, 1997) and the senescence of carnation flowers (Chapter 4) by ethanol has been attributed to the formation of acetaldehyde within treated tissues. The application of acetaldehyde as a post-harvest treatment resulted in very similar

longevity increases to those seen after ethanol application. When ethanol was applied to cut carnation flowers a number of distinct physiological responses were observed. Ethylene formation was completely inhibited (HEINS, 1980; WU, ZACHARIAS, SALTVEIT and REID, 1992; Chapters 2 and 3). Ovary development was inhibited (HEINS and BLAKELY, 1980; Chapter 3), and petal in-rolling was replaced by petal browning (Chapter 2). Chlorophyll content of the stem, calyx and ovary decreased. The carbohydrate status of the flowers was particularly disrupted. Losses were recorded in carbohydrate content of all the organs of the flower. The source sink activity of the ovary and petals was completely interrupted. Ethanol also resulted in decreased protein content in all plant parts. This was due to a total, rather than specific, protein loss. The inhibition of ethylene formation was due to the inactivation of the enzyme ACC oxidase, as well as the prevention of the formation of ACC itself (Chapter 3).

Acetaldehyde has the ability to promote and inhibit fruit ripening (BEAULIEU and SALTVEIT, 1997) and flower senescence (Chapter 5). This is a concentration-related response, as acetaldehyde only promotes ripening and senescence when applied at a low concentration. For the purpose of this study we were only interested in the prevention of flower senescence, and the resultant extension of vase life. The aim of the experimentation performed in this Chapter was to determine the physiological response of cut carnation flowers to acetaldehyde.

6.2. Materials and methods

Apart from the use of acetaldehyde, rather than ethanol, as a holding solution, the materials and methods for the experimentation performed in this Chapter were

exactly the same as those for Chapter 3. Investigations into the effect of acetaldehyde on dry weight, moisture content, chlorophyll content, carbohydrate status, protein content, ACC content and ACC oxidase activity were performed. Standard curves for ethylene, sugars, protein, ACC and ACC oxidase can be found in Chapters 2 and 3. The investigation into the movement of applied ethanol was not performed.

6.3. Results

6.3.1. The effect of acetaldehyde treatment on the physical appearance of cut carnation flowers

The physical response of flowers to acetaldehyde treatment was very similar to that of ethanol. Petal in-rolling did not occur. Instead severe petal browning resulted from acetaldehyde treatment. This petal browning was more severe than that seen in ethanol-treated flowers (PLATE 6.1.). The enlarging and greening of the ovary was also inhibited (PLATE 6.2.). The effect again seemed to be more severe in the acetaldehyde-treated flowers.

The effect of acetaldehyde on the dry weight and moisture content of the flowers was determined. Flowers in these experiments were treated with a number of concentrations of acetaldehyde. Their resultant longevity is presented in TABLE 6.1. The effect of acetaldehyde treatment on the dry weight of petals of cut carnation flowers is presented in TABLE 6.2. The effect on the moisture content is shown in TABLE 6.3.

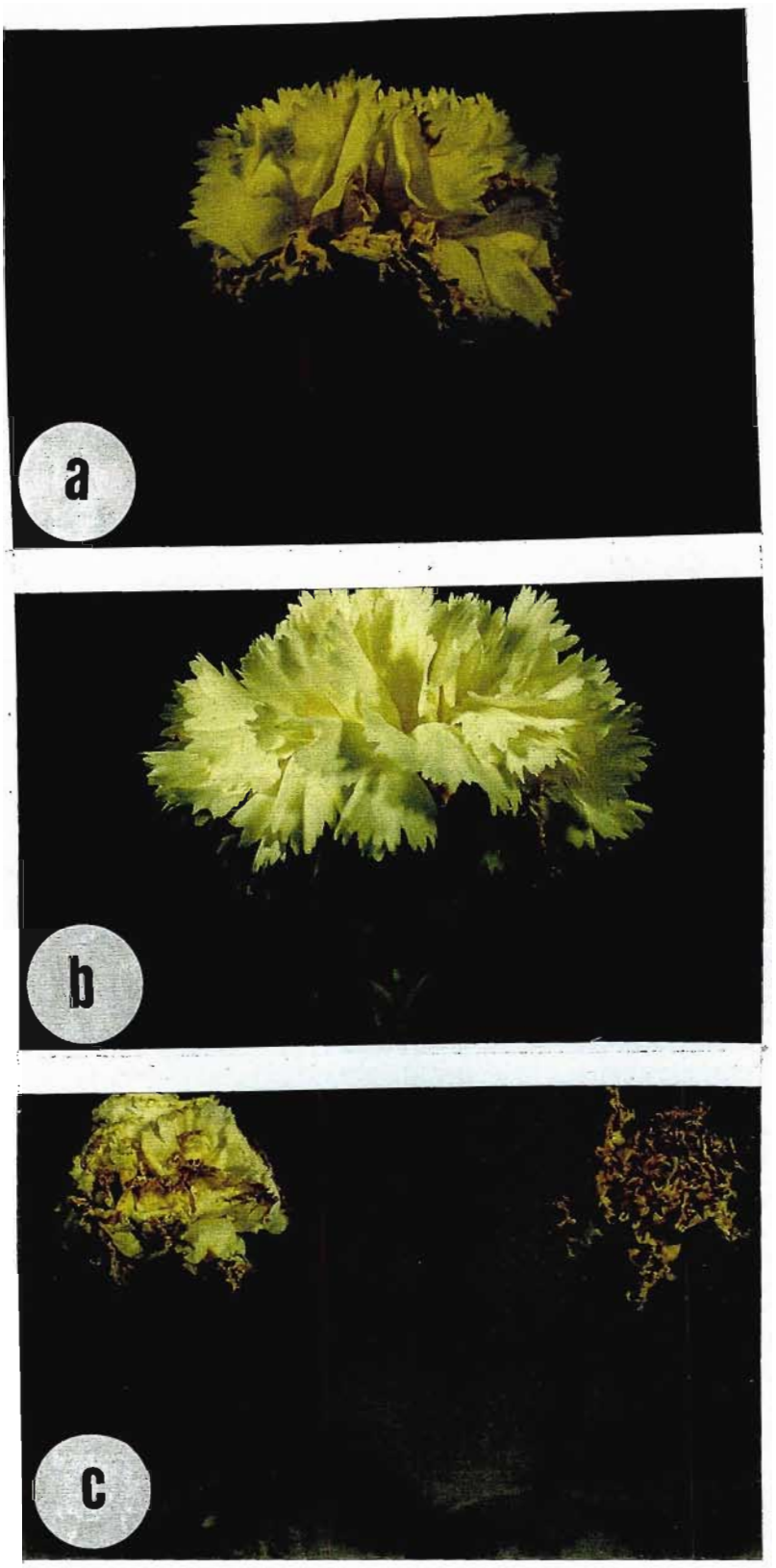


Plate 6.1. The appearance of cut carnation flowers after 9 days. A = flowers treated with acetaldehyde, B = flowers treated with ethanol and C = control flowers. Note the severity of petal burning in acetaldehyde-treated flowers compared to the ethanol-treated flowers.

Plate 6.2. Ovaries of flowers treated with water, 3% ethanol (3% EtOH) and 2% acetaldehyde (2% AcA).

TABLE 6.1. The longevity (days) of the flowers used in the moisture content experiments, which were treated with various concentrations of acetaldehyde.

	Acetaldehyde concentration (%)						
	0	1	1.5	2	2.5	3	4
S50 (days)	7.5	10.2	12.2	12.4	12.2	12.1	11.4

Table 6.2. The effect of acetaldehyde on the dry weight (g) of the petals of cut carnation flowers. Standard errors are indicated in brackets. Results with the same letters are not significantly different from one another.

Time after the onset of treatment (days)	Acetaldehyde concentration (%)						
	0	1	1.5	2	2.5	3	4
0	1.0421 (0.087a)	1.0421 (0.087a)	1.0421 (0.087a)	1.0421 (0.087a)	1.0421 (0.087a)	1.0421 (0.087a)	1.0421 (0.087a)
4	0.916 (0.134a)	1.067 (0.172a)	1.084 (0.395a)	1.473 (0.53a)	0.826 (0.208a)	1.102 (0.18a)	1.189 (0.1a)
8	0.838 (0.19ab)	1.009 (0.177a)	0.828 (0.072b)	0.974 (0.07ab)	0.791 (0.082b)	0.866 (0.031b)	0.866 (0.068b)
12	0.663 (0.045a)	0.814 (0.104b)	0.883 (0.041b)	0.868 (0.02b)	0.844 (0.038b)	0.848 (0.102b)	0.842 (0.072b)

Table 6.3. The effect of various concentrations of acetaldehyde on the moisture content (%) of the petals of cut carnation flowers

Time after the onset of treatment (days)	Acetaldehyde concentration (%)						
	0	1	1.5	2	2.5	3	4
0	84.9	84.9	84.9	84.9	84.9	84.9	84.9
4	85.6	78.4	79.9	82.2	84.6	81.4	82.2
8	83.5	80.7	82.7	83.4	82.4	83.7	83.9
12	50.2	76.3	70.5	79.2	80.3	81.1	80.4

As the petals of control flowers rolled inwards, so their dry weight gradually decreased. The moisture content however, remained at a constant level until the last day of the experiment. At this point the control flowers had been fully senesced for several days, and the decrease in moisture content was due to the final stages of senescence. In treated flowers petal browning occurred, and the gradual loss of dry weight was not seen. Instead both the dry weight and the moisture content remained constant throughout the experiment. Even twelve days after harvest the petals still had a moisture content of 80%.

The effect of acetaldehyde treatment on the dry weight (TABLE 6.4) and moisture content (TABLE 6.5) of the ovaries of acetaldehyde-treated cut carnation flowers was determined. The dry weight of the control ovaries increased throughout the experimental period, due to their activity as a carbohydrate sink. Their moisture content, like that of the petals, remained constant. The ovaries of flowers treated with acetaldehyde did not gain dry weight. In fact they lost significant amounts of weight, and an increase in the concentration of acetaldehyde applied did not result in a

significant change in the amount of dry weight lost. The moisture content did not change at all as a result of acetaldehyde treatment.

Table 6.4. The effect of acetaldehyde on the dry weight (g) of ovaries of cut carnation flowers. Standard errors are indicated in brackets. Results with the same letters are not significantly different from one another.

Time after the onset of treatment (days)	Acetaldehyde concentration (%)						
	0	1	1.5	2	2.5	3	4
0	0.087 (0.184a)	0.087 (0.184a)	0.087 (0.184a)	0.087 (0.184a)	0.087 (0.184a)	0.087 (0.184a)	0.087 (0.184a)
4	0.097 (0.036a)	0.084 (0.019a)	0.054 (0.015b)	0.073 (0.02ab)	0.0487 (0.01b)	0.06 (0.03ab)	0.055 (0.01ab)
8	0.105 (0.024a)	0.061 (0.019b)	0.051 (0.017b)	0.051 (0.006b)	0.059 (0.005b)	0.055 (0.013b)	0.053 (0.016b)
12	0.114 (0.012a)	0.044 (0.019b)	0.046 (0.009b)	0.049 (0.017b)	0.041 (0.004b)	0.042 (0.067b)	0.048 (0.008b)

Table 6.5. The effect of various concentrations of acetaldehyde on the moisture content (%) of ovaries of cut carnation flowers.

Time after the onset of treatment	Acetaldehyde concentration (%)						
	0	1	1.5	2	2.5	3	4
0	85.4	85.4	85.4	85.4	85.4	85.4	85.4
4	81.7	81.0	85.0	80.2	83.4	83.8	83.6
8	83.2	84.2	84.3	84.9	84.7	81.6	82.5
12	83.2	85.6	86.1	87.3	86.0	86.4	84.2

The stems, calyxes and ovaries of flowers treated with acetaldehyde showed severe discolouration (PLATE 6.3.). In particular the ovaries, which should have become a darker green colour during the senescence process, were yellow in appearance. The effect of various concentrations of acetaldehyde on the total chlorophyll content of the ovary, stem and calyx of cut carnation flowers was determined (FIGURE 6.1.). In control flowers the chlorophyll content of the stem and calyx remained constant throughout the vase life. The chlorophyll content of the ovary increased significantly during the same period. The acetaldehyde treatments resulted in a reduction in the chlorophyll content of all of the organs tested. Levels in the stem and the ovary were significantly reduced compared to the control flowers. By the end of the experiment as much as 50% of the chlorophyll present in the ovaries of flowers at the time of harvesting had disappeared in ovaries of flowers treated with 3% acetaldehyde. The response seen in the treated flowers was the same regardless of the concentration of acetaldehyde applied, indicating that no concentration effect existed. It appeared that acetaldehyde treatment, led to the degeneration of chloroplasts and loss of membrane integrity.

6.3.2. The effect of acetaldehyde treatment on carbohydrate translocation and accumulation

Total carbohydrate levels were determined in the stems, petals and ovaries of flowers treated with 1.5% acetaldehyde (FIGURE 6.2.). In petals of control and treated flowers the total carbohydrate content decreased substantially throughout the experimental period. In control flowers the decrease in carbohydrates was as a result of the petals acting as a carbohydrate source within the system. The rate of carbohydrate decrease was significantly greater in

Plate 6.3. The appearance of calyxes and ovaries treated with acetaldehyde, indicating the decrease in greenness. Test boxes indicate the concentration of acetaldehyde applied.

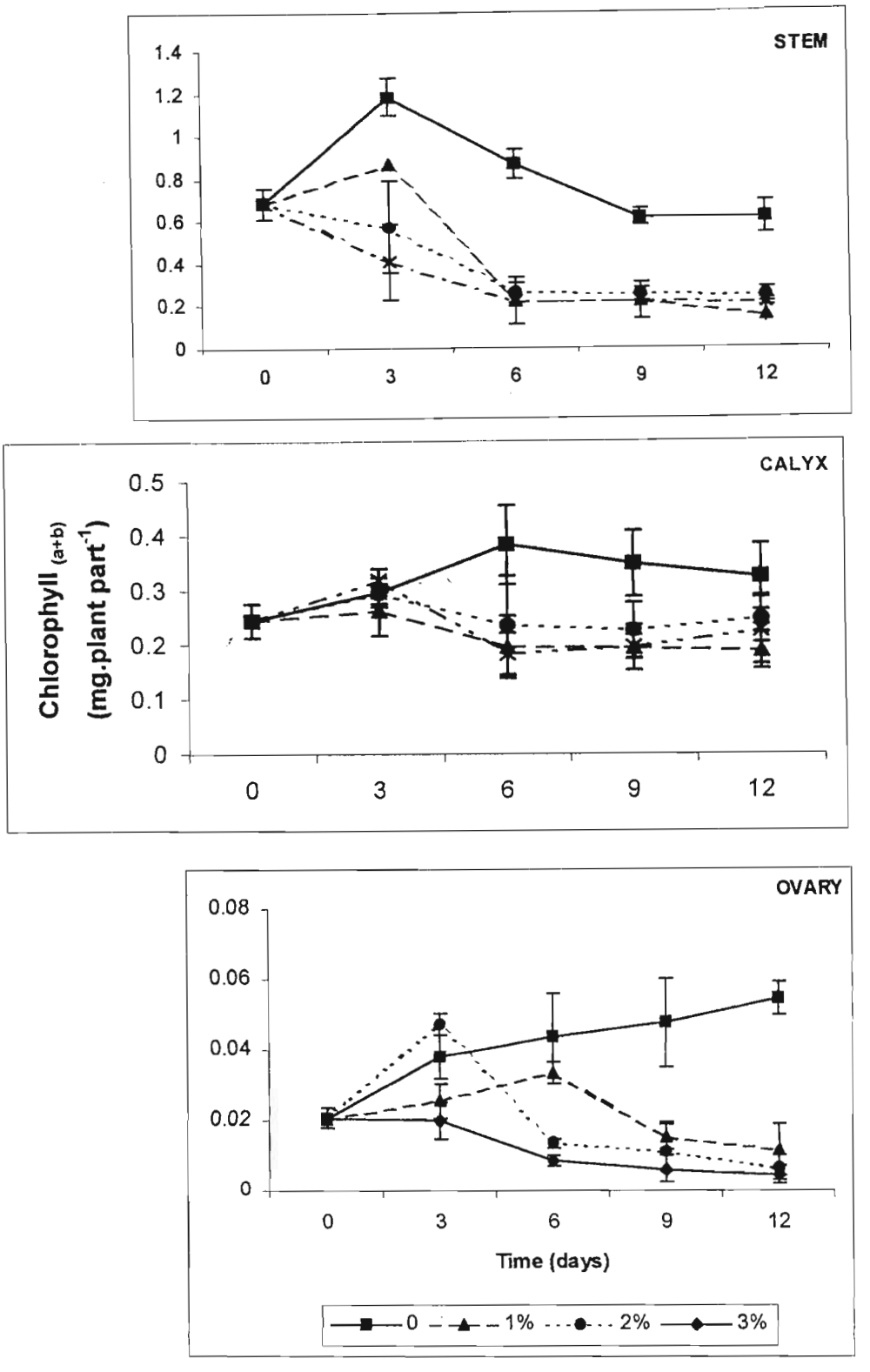


Figure 6.1. The effect of acetaldehyde on the chlorophyll content of stems, calyxes and ovaries. Bars indicate standard errors. Note that axes are not the same for the stem, calyx and ovary.

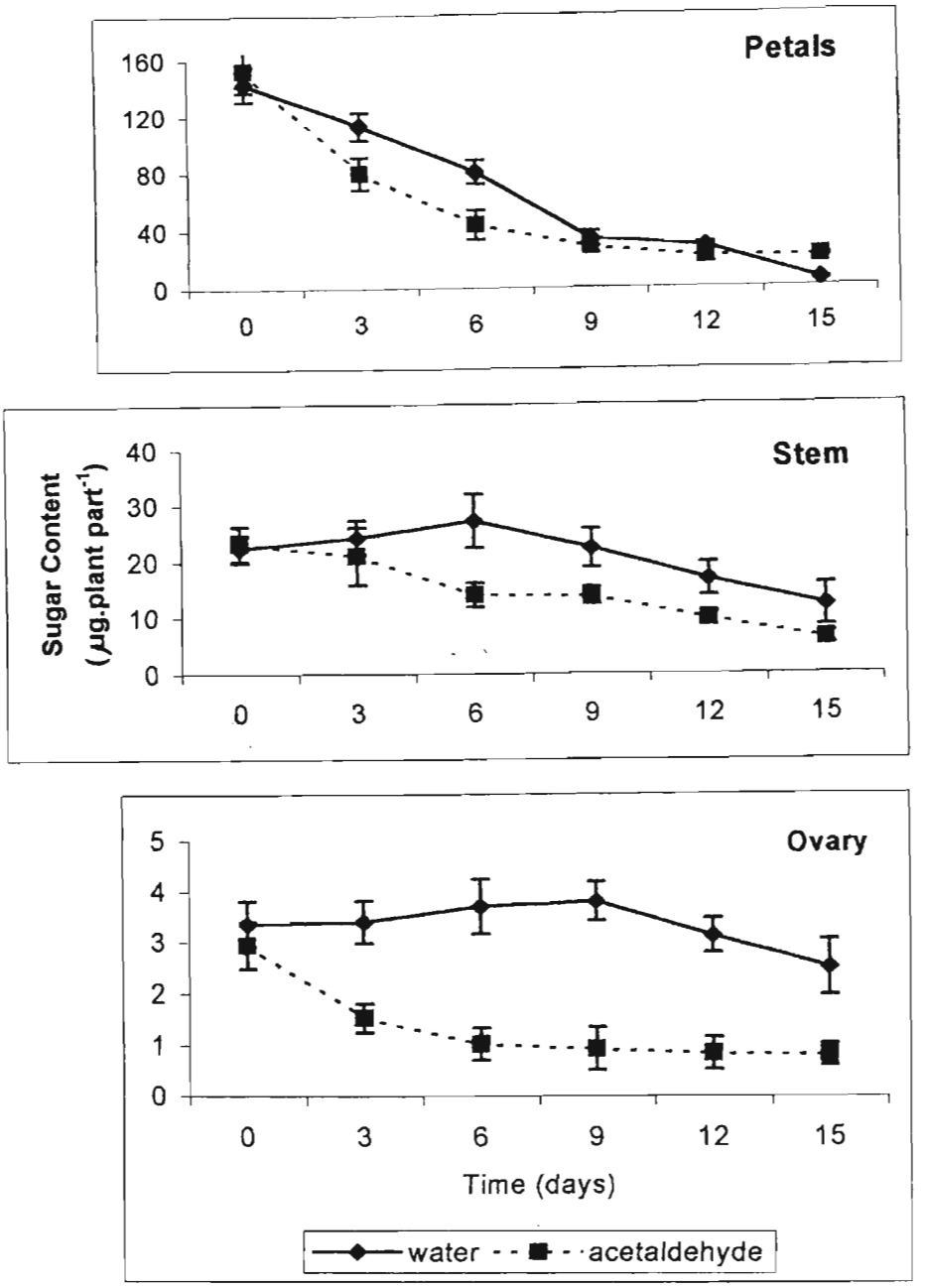


Figure 6.2. The effect of 1.5% acetaldehyde on the total carbohydrate content of cut carnation flowers. Note that axes are not the same for petals, stems and ovaries. Bars indicate standard errors.

acetaldehyde-treated flowers than the control flowers. This indicates that acetaldehyde treatment disturbs the normal carbohydrate status of senescing petals. In the stem of control flowers there was an initial minor increase in carbohydrates as the reserves were mobilized, after which levels decreased. In acetaldehyde-treated petals there was no initial increase in total carbohydrate content. Instead there was a gradual decline throughout the experimental period. The greatest effect on the total carbohydrate content was seen in extracts from the ovaries. In control flowers there was an increase in total carbohydrate levels as the ovary acted as a carbohydrate sink within the system. In the treated ovaries no increase in carbohydrate content was seen. There was a dramatic decrease in the carbohydrate content of ovaries of flowers treated with acetaldehyde. This indicates that acetaldehyde inhibits the action of the ovary as a carbohydrate sink within cut carnation flowers.

Throughout the senescence process the monosaccharide levels in the petals and stems were almost identical between flowers treated with 1.5% acetaldehyde and the controls (FIGURE 6.3.). In the ovary, levels were the same in both experimental groups for the first three days of the experiment. After this however, the monosaccharide levels decreased dramatically in acetaldehyde-treated flowers, while in the water control levels increased due to the sink activity.

Sucrose was the only disaccharide carbohydrate detected in the flowers. The effect of acetaldehyde on its levels are shown in FIGURE 6.4. In the petals of untreated flowers the sucrose level dropped immediately after harvesting. This effect was also seen in the acetaldehyde-treated flowers. In the stems and ovaries

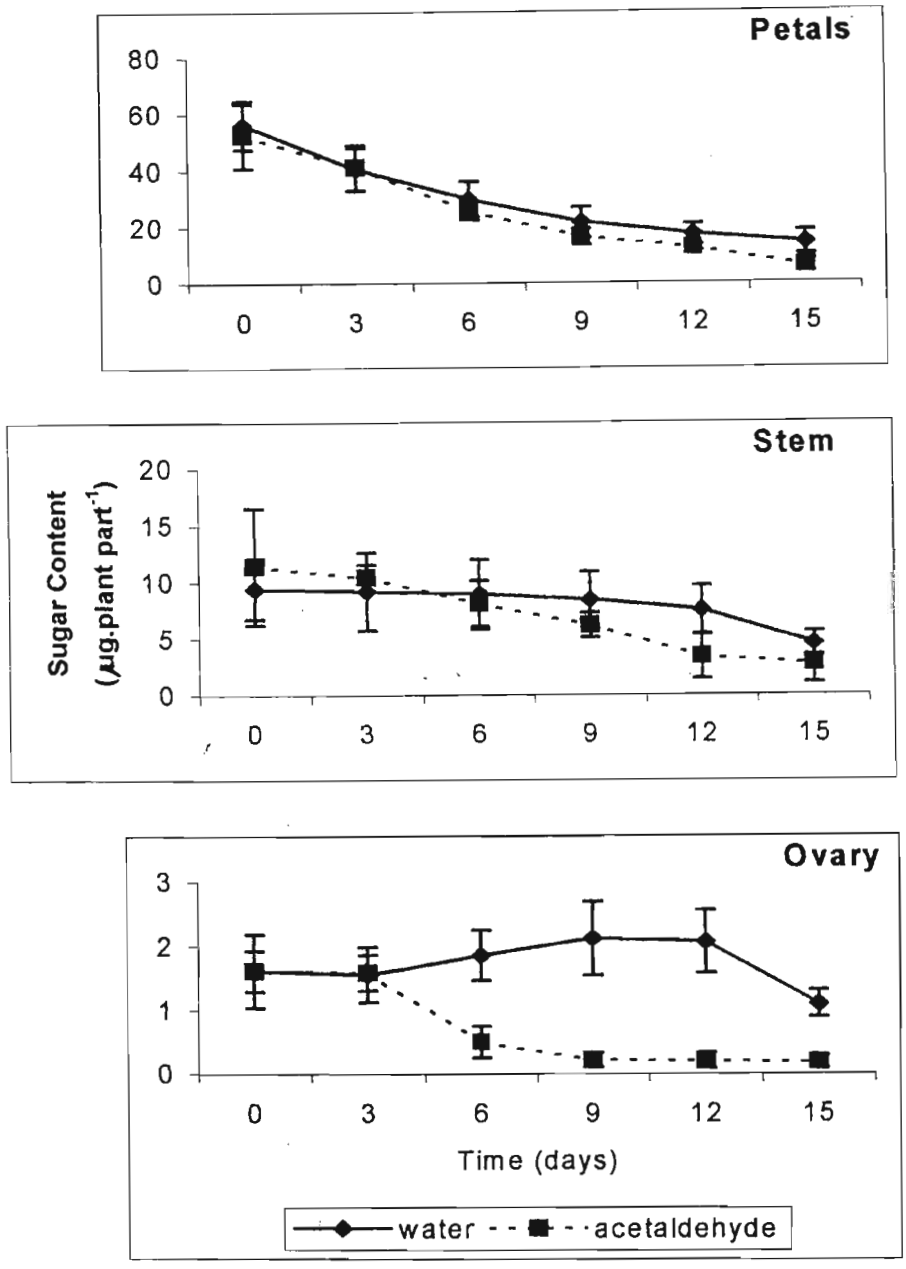


Figure 6.3. The effect of 1.5% acetaldehyde on monosaccharide sugar content of cut carnation flowers. Note that the axes are not the same for petals, stems and ovaries. Bars indicate standard errors.

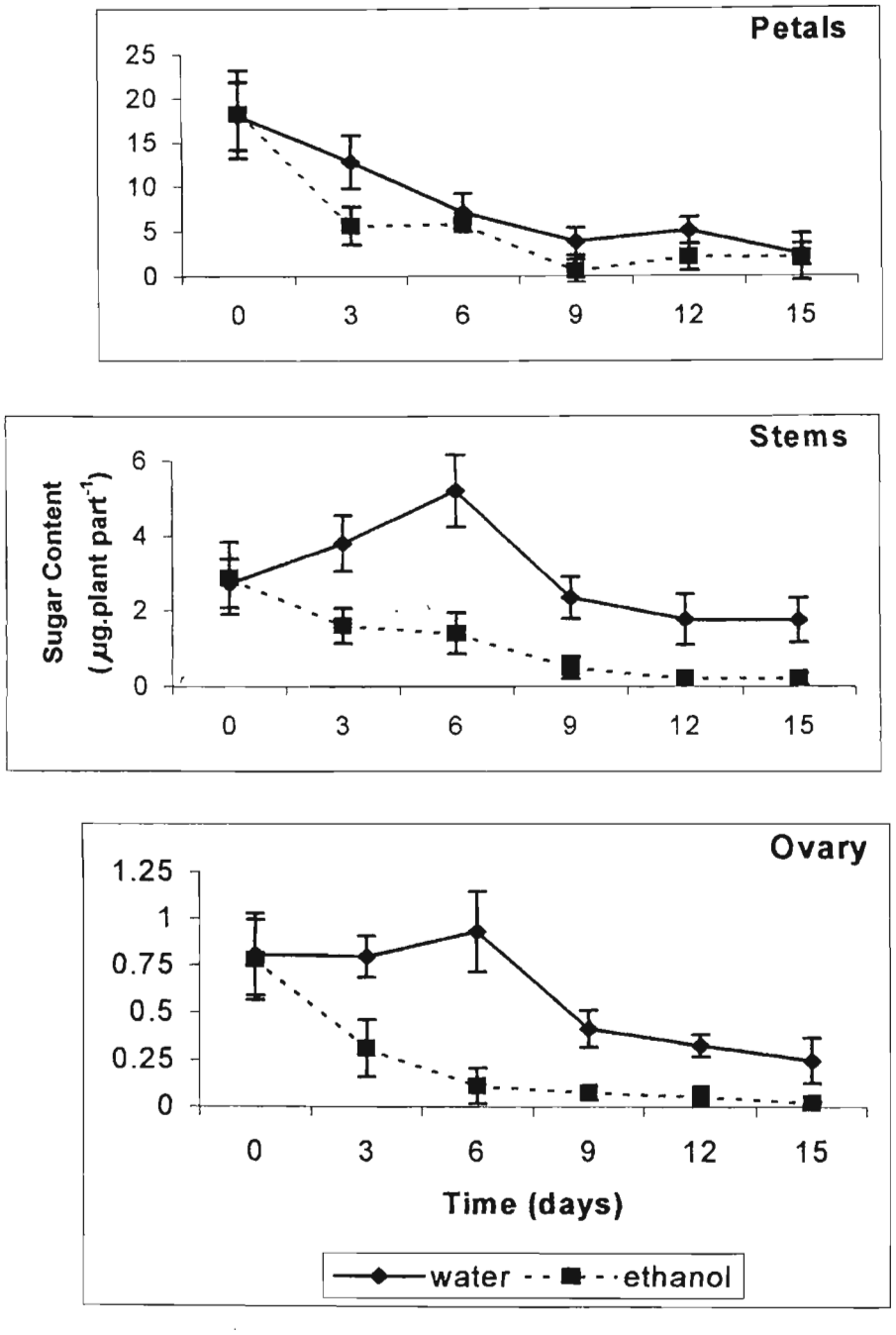


Figure 6.4. The effect of 1.5% acetaldehyde on sucrose content of cut carnation flowers. Note that axes are not the same for petals, stems and ovaries. Bars indicate standard errors.

however, the sucrose level increased until the sixth day of the experiment, as the ovary mobilized carbohydrates, via the receptacle, to facilitate development. After the sixth day sucrose levels declined gradually, as a result of utilization of the carbohydrates for cellular processes. This trend was not seen in acetaldehyde-treated flowers. In the stems and ovaries of treated flowers, like the petals, the sucrose levels were observed to decrease dramatically after the onset of treatment, and remain at significantly reduced levels compared to the water control throughout the vase life of the flowers.

Several sugar alcohols were detected in the tissues of senescing carnation flowers (inositol, mannitol and sorbitol). In the petals and stems of the control flowers the levels of these sugar alcohols decreased throughout the experimental period (FIGURE 6.5). In the ovary there was an increase in sugar alcohol concentration until the ninth day of treatment, after which it decreased. The concentration of sugar alcohols in all organs of acetaldehyde-treated flowers decreased, not just in the stems and calyx, but also in the ovary. This decrease began immediately after the onset of acetaldehyde treatment, and the levels remained low throughout flower vase life.

The application ^{14}C sucrose to acetaldehyde-treated flowers resulted in a clear pattern (FIGURE 6.6.). For both the treated and control flowers 50% of the ^{14}C remained in the marked petal, or was not absorbed, as this reading included the ^{14}C that was washed off the petals. Of the radioactivity that was absorbed by the control flowers, the majority was recovered in the ovaries. This indicated that the ovary indeed acted as a carbohydrate sink, and the petals as a source. Almost no

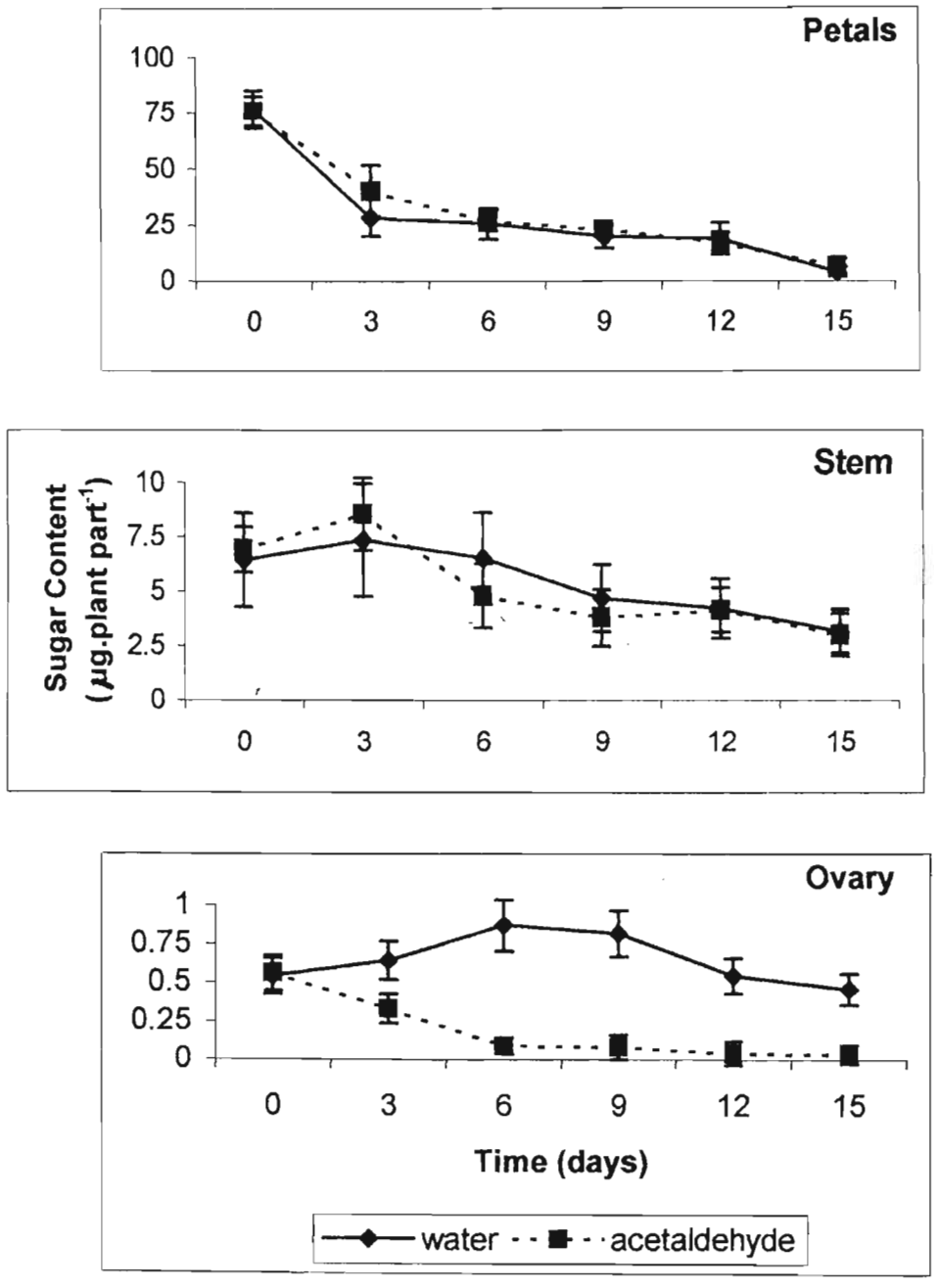


Figure 6.5. The effect of 1.5% acetaldehyde on sugar alcohol (inositol, mannitol and sorbitol) levels of cut carnation flowers. Note that the axes are not the same for petals, stems and ovaries. Bars indicate standard errors.

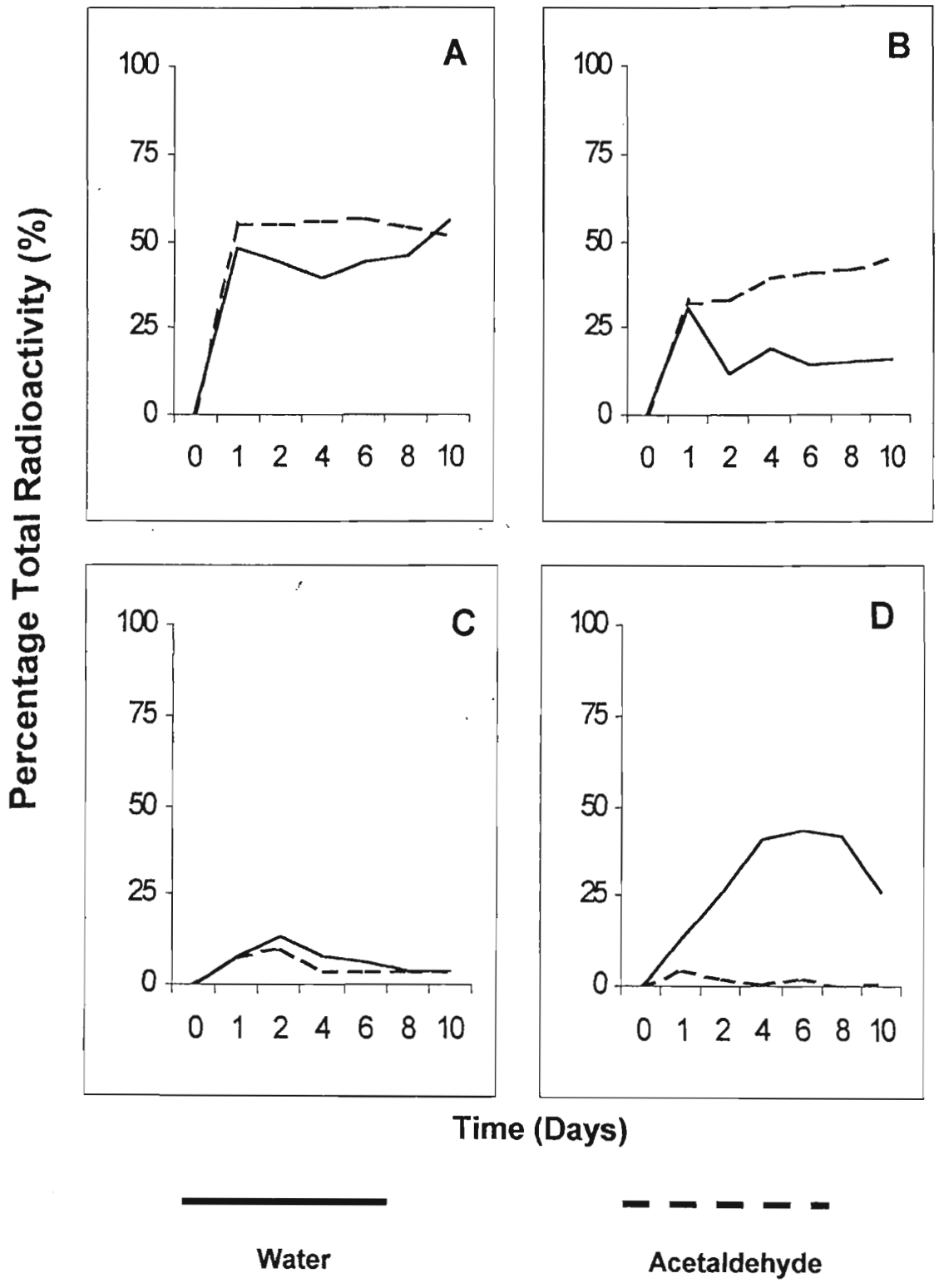


Figure 6.6. The percentage of applied ^{14}C (sucrose), recovered in the various organs of acetaldehyde-treated flowers. A = marked petal; B = untreated petals; C = stem; D = ovary.

radioactivity was recorded in the ovaries of flowers treated with acetaldehyde however. A small amount of ^{14}C was recovered from the stems of treated flowers on the first and second days of the experiment. All of the remaining radioactivity was found in the petals. It is clear that acetaldehyde negates the effect of the ovary as a carbohydrate sink within the system, and the carbohydrates remain within the petal tissues of treated flower.

6.3.3. The effect of acetaldehyde treatment on the protein content of cut carnation flowers

Proteins are essential for cellular metabolism and functioning. The protein content of the petals and ovaries of acetaldehyde-treated flowers was determined to establish if the treatment altered the protein content of the organs. The longevity of the flowers used in this study differed to the longevity of previous flowers, and is recorded in TABLE 6.6.

Table 6.6. Longevity of flowers used for protein determination, treated with either water or 1.5% acetaldehyde.

	Acetaldehyde Concentration (%)	
	0	**1.5**
S50 (days)	5	9

The protein content of the petals of acetaldehyde-treated flowers was greater than the concentration of protein present in the petals of control flowers throughout the experimental period when protein was expressed on per plant part basis (FIGURE 6.7). However, at this point the fresh weight of the petals of the treated

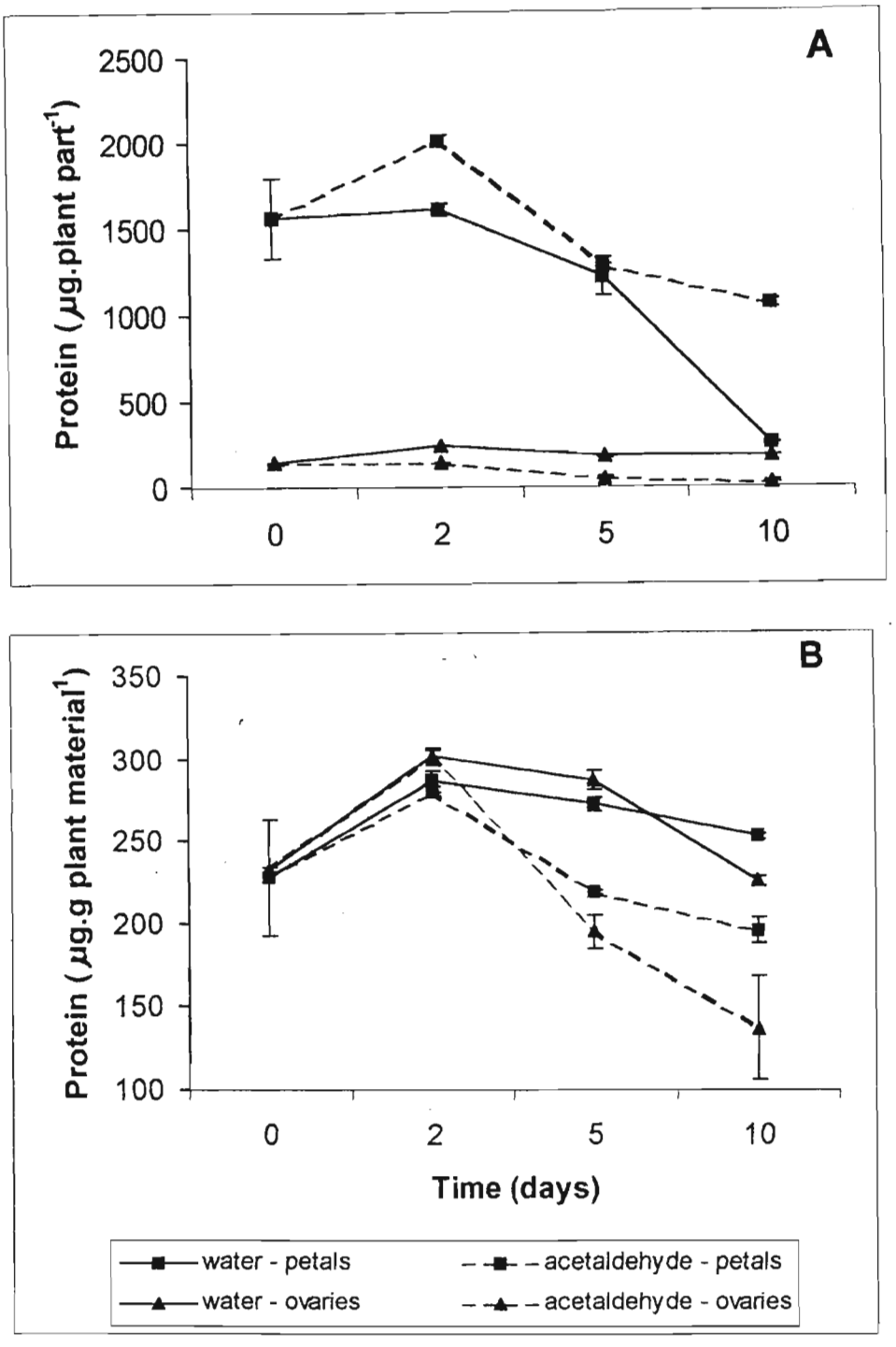


Figure 6.7. The effect of 1.5% acetaldehyde on the protein content of petals and ovaries of cut carnation flowers. A = results presented as µg.plant part^{-1}; B = results presented as µg.g plant material^{-1}. Bars indicate standard errors.

flowers was much greater than the control flowers, which were 50% senesced on the fifth day of the experiment. The same data was analyzed as µg protein.g plant material $^{-1}$, removing the effect of moisture. The amount of protein in the control petals was then much higher than in treated flowers, indicating that protein loss had occurred in treated flowers. In the ovaries of flowers treated with 1% acetaldehyde there was a significant reduction in the protein content, regardless of the presentation of the data. Acetaldehyde treatment resulted in a decrease in the protein content of cells of cut carnation flowers.

It was necessary to determine whether the protein losses were as a result of the targeting of specific enzymes, or as a indiscriminant loss of total protein content from the cells. This was done by monitoring the visual changes in the proteins of treated and control organs throughout the experimental period, making use of SDS-PAGE gels. Protein extracts from the second, fifth and tenth days of acetaldehyde treatment were separated out by size and compared to control flowers. On the second day of the experiment, the pattern and density of protein separation was similar between treated and untreated flowers (FIGURE 6.8). In the petal extracts of treated flowers a group of polypeptides, between 20.1 and 26.6 kDa in size, had decreased in density. In the same extracts a protein band slightly smaller than 20.1 kDa in size increased in treated petals. In the ovaries, two groups of polypeptides decreased as a result of acetaldehyde treatment. The first was the same group that had deceased in the petals, the other was a group of larger proteins, between 55.6 and 116.4 kDa in size. By the fifth day of the experiment a notable decrease in the density of bands of proteins from treated flowers was observed (FIGURE 6.9). In the petals the group of proteins that was reduced on the second day of the experiments

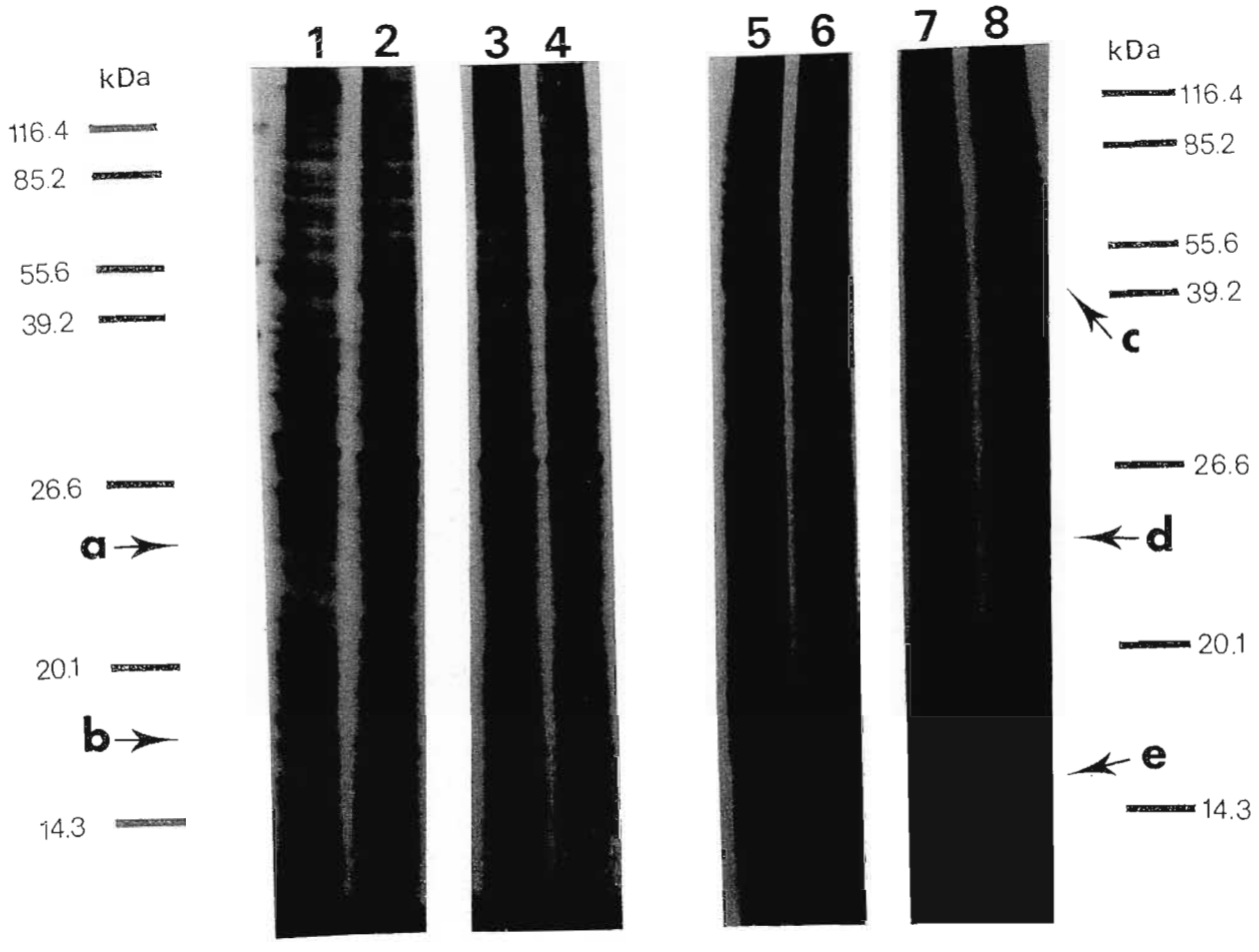


Figure 6.8. SDS-PAGE separation of proteins of control and acetaldehyde- treated flowers on the second day of treatment. Lanes 1 and 2 contain proteins extracted from control petals, lanes 3 and 4 contain proteins extracted from petals treated with 1% acetaldehyde, lanes 5 and 6 contain proteins extracted from control ovaries, and lanes 7 and 8 contain proteins extracted from ovaries treated with 1.5% acetaldehyde. The polypeptides group labeled A have decreased in petals of treated flowers compared to the control. The band labeled B increased. Those labeled C, D and E have decreased in ovaries of treated flowers compared to the control.

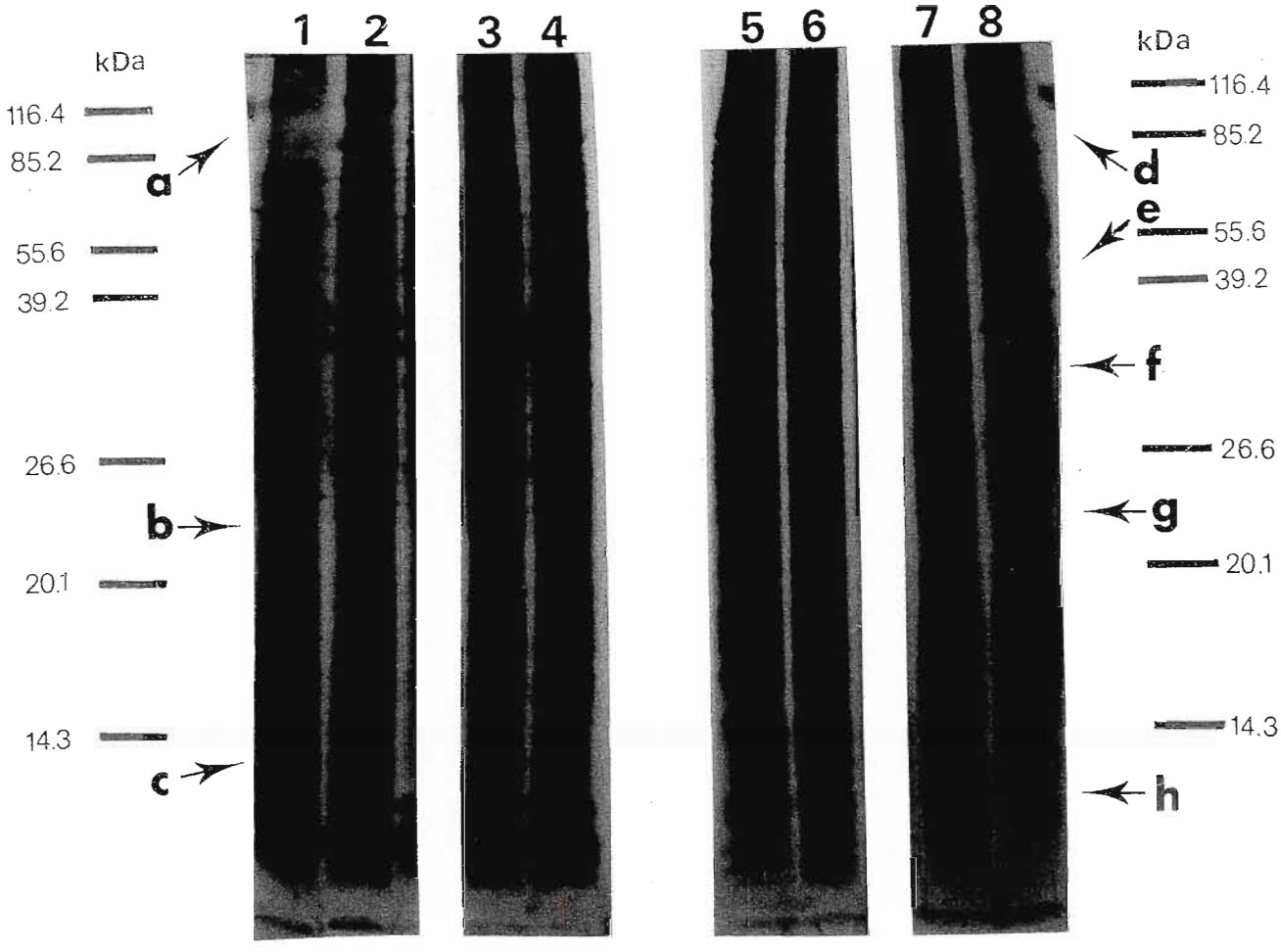


Figure 6.9. SDS-PAGE separation of proteins of control and acetaldehyde- treated flowers on the fifth day of treatment. Lanes 1 and 2 contain proteins extracted from control petals, lanes 3 and 4 contain proteins extracted from petals treated with 1.5% acetaldehyde, lanes 5 and 6 contain proteins extracted from control ovaries, and lanes 7 and 8 contain proteins extracted from ovaries treated with 1% acetaldehyde. The polypeptides groups labeled A, B and C have decreased in petals of treated flowers compared to the control. Those labeled D, E, F, G and H have decreased in ovaries of treated flowers compared to the control.

(between 26.6 and 20.3 kDa) was again less dense than in the control flowers. A group of very large proteins (around 70 – 100 kDa) and a group of very small proteins (less than 14.3 kDa) were also notably decreased. The band that increased on day 2 did not increase further. In the ovaries a number of groups of proteins, ranging from very small to very large, were observed to be much lower in the extract from treated flowers. In fact, in the eighth lane the proteins appeared smeared, and did not separate clearly into bands, indicating severe protein damage. On the tenth day of experimentation there was almost no definite band formation at all in the protein extracts from flowers treated with acetaldehyde (FIGURE 6.10). This indicated that severe protein breakdown had occurred in both the ovaries and the petals of the treated flowers. The visual results were confirmed by the results seen in the protein concentration experiments, indicating that acetaldehyde treatment resulted in loss and damage to the proteins of cells of cut carnation flowers. This breakdown is a general phenomenon for all the polypeptides, and is not restricted to certain enzymes within the cells.

6.3.4. The effect of acetaldehyde treatment on ethylene formation

Two parts of the ethylene biosynthetic pathway were investigated: the effect of acetaldehyde on ACC formation, and its effect on ACC oxidase activity. The effect of a 1.5% acetaldehyde treatment on ethylene formation was investigated, and compared to a water control. The longevity of both treatments is presented in TABLE 6.7. The ethylene production of the flowers was monitored (FIGURE 6.11.). As earlier results revealed, ethylene formation was almost totally inhibited by acetaldehyde treatment (Chapter 5). The formation of ACC within the system was markedly reduced by acetaldehyde (FIGURE 6.12). In control flowers, as climacteric

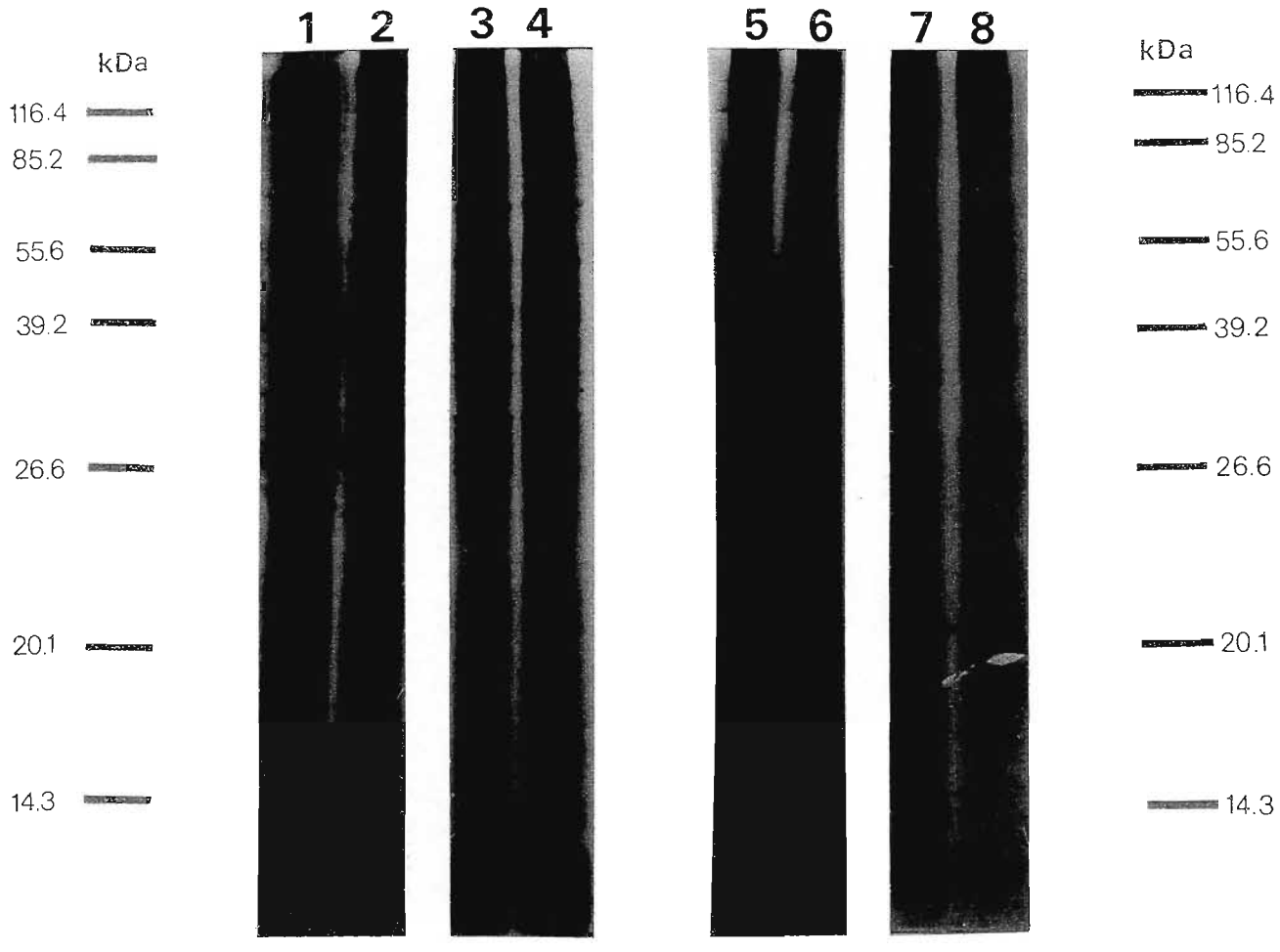


Figure 6.10. SDS-PAGE separation of proteins of control and acetaldehyde-treated flowers on the tenth day of treatment. Lanes 1 and 2 contain proteins extracted from control petals, lanes 3 and 4 contain proteins extracted from petals treated with 1.5% acetaldehyde, lanes 5 and 6 contain proteins extracted from control ovaries, and lanes 7 and 8 contain proteins extracted from ovaries treated with 1% acetaldehyde. Very few bands are discernible in both the ovaries and petals of acetaldehyde-treated flowers, indicating general protein loss.

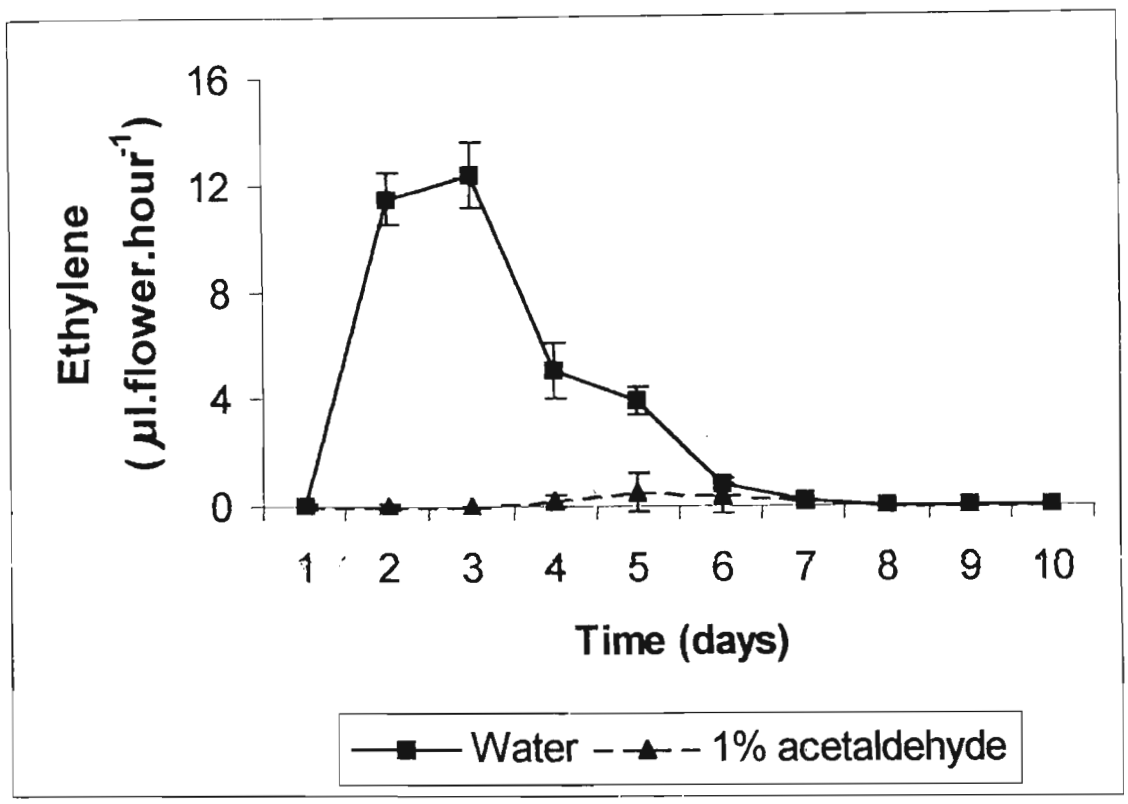


Figure 6.11. Ethylene production by cut carnation flowers treated with acetaldehyde, which were used for the determination of ACC content and ACC oxidase activity. Bars indicate standard errors.

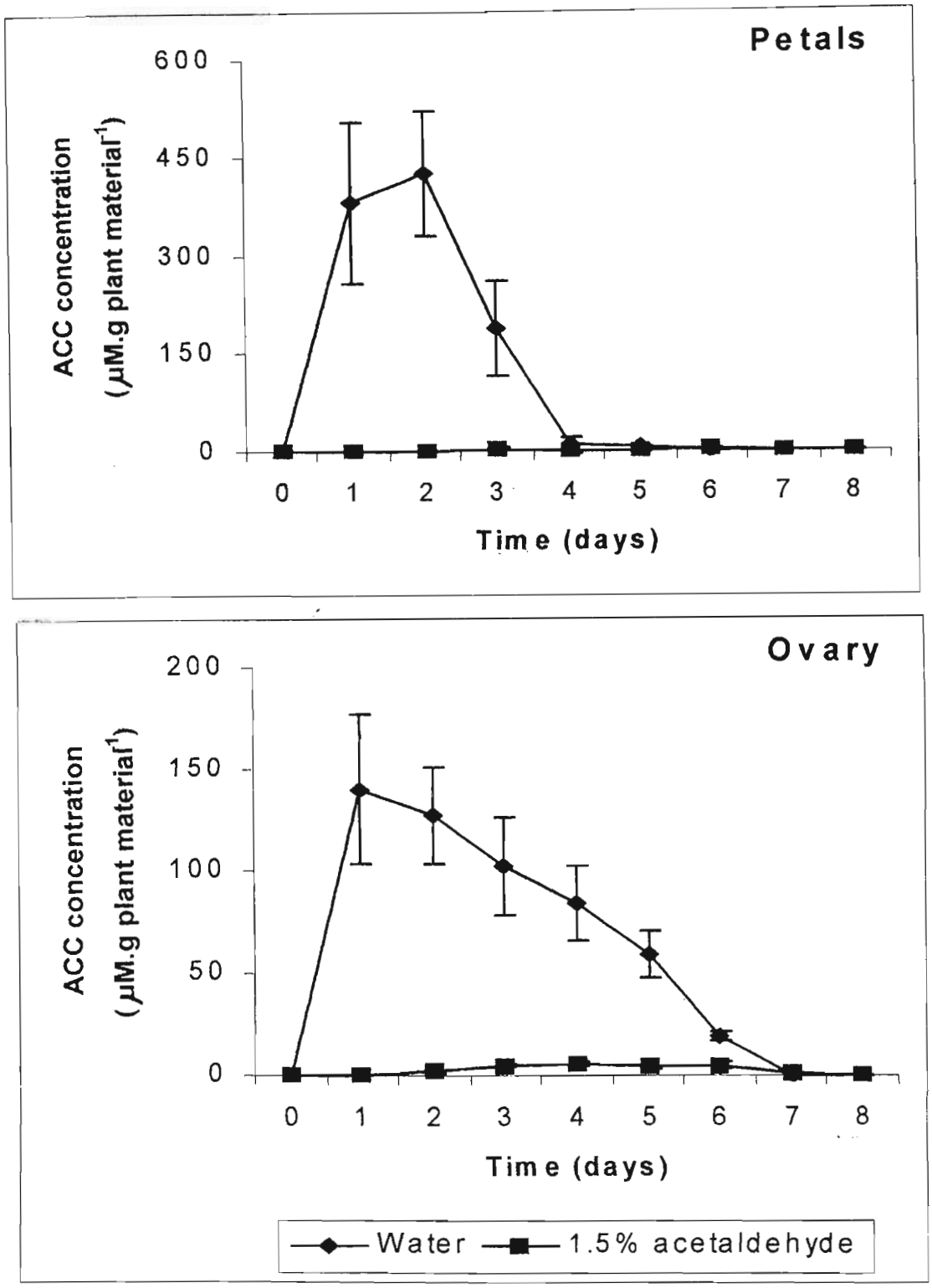


Figure 6.12. ACC content of flowers treated with 1.5% acetaldehyde. Bars indicate standard errors.

ethylene production occurred, huge increases in ACC content were recorded, particularly in the petals. However, in acetaldehyde-treated flowers no ACC was found in the petal or ovary tissues of flowers at any point during the experimental period. ACC oxidase activity within the extracts was also completely prevented in the petals and ovaries by the application of acetaldehyde to the flowers (FIGURE 6.13). Therefore, no ACC was allowed to form within treated tissues, and even if it had, the conversion to ethylene would not had been able to occur and ethylene production would still have been entirely prevented.

Table 6.7. Longevity of flowers used for the investigation into the effect of acetaldehyde on ethylene formation. Flowers were treated with either water or 1.5% acetaldehyde.

	Acetaldehyde Concentration (%)	
	0	**1.5**
Longevity (S50)	7	10

6.4. Discussion

Acetaldehyde treatment resulted in a number of physiological responses in cut carnation flowers. Amongst these were the prevention of ovary development, the disruption of the source sink activity between the petals and ovary, a decrease in the protein content of petal and ovary cells, and complete inhibition of ethylene formation.

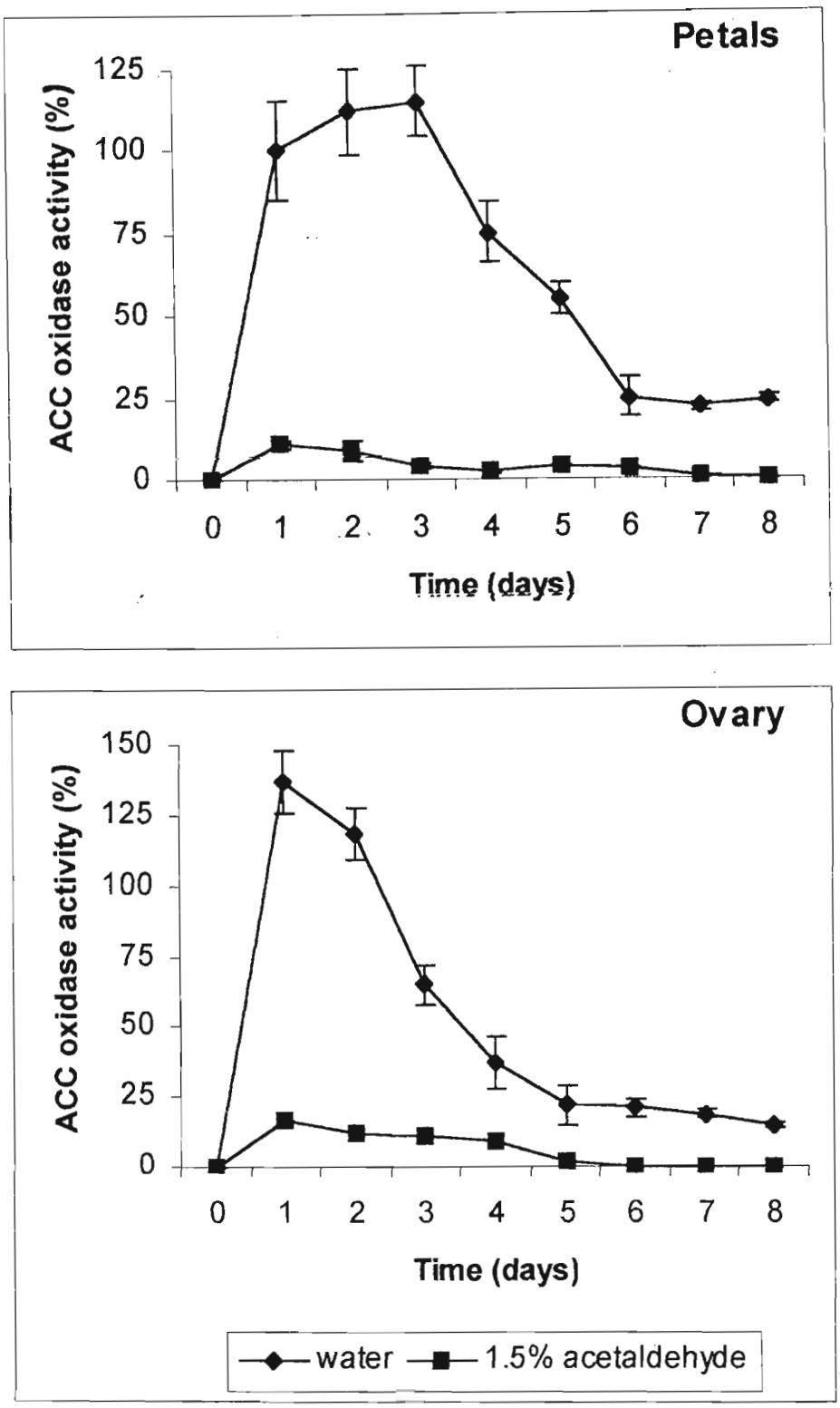


Figure 6.13. ACC oxidase activity in extracts from cut carnation flowers treated with 1.5% acetaldehyde. Bars indicate standard errors.

The ovary is the site of seed set in flowers. As a result, as the rest of the flower dies, the ovary of cut carnation flowers should develop and grow. The ovary acts as a carbohydrate sink within the system, resulting in carbohydrates being mobilized from the petals of the flowers, and moving via the receptacle to the ovary (NICHOLS 1977a; HALEVY and MAYAK, 1979; DIMALLA and VAN STADEN, 1980; DUCASSE and VAN STADEN, 1981; COOK and VAN STADEN, 1983). As a result of ethylene formation, the development of the ovary increases, and the chloroplasts exhibit growth and so the chlorophyll content of the tissues increases significantly (CAMPURBI and NICHOLS, 1979; COOK and VAN STADEN, 1983; 1986). Acetaldehyde inhibited all of these processes. All concentrations of acetaldehyde above 1% inhibited ethylene formation entirely, by preventing ACC formation and ACC oxidase activity. In response to this, many of the ethylene associated senescence characteristics of senescing carnations were not manifested.

Petal in rolling did not occur, instead petal browning was observed. The dry weight of the petals decreased at a reduced rate to those of the control petals, while the moisture content remained constant throughout the entire vase life of the flowers. Although dry weight losses were recorded, they were not to the same extent as the control flowers. The carbohydrate content was determined in the petals and ovaries. In the petals of control and treated flowers there was a huge decrease in all the carbohydrates recorded. The losses in the control petals were due to their role as a carbohydrate source within the flower. It appears that acetaldehyde prevents this process. This was confirmed when the movement of ^{14}C sucrose applied to a petal of an acetaldehyde-treated flower was monitored. Unlike the control flowers, where 50% of the radioactivity was found in the ovary, almost no ^{14}C was found in treated

ovaries. Instead the ^{14}C was found to be in the other petals, or not to have moved from the treated petal at all. Although the carbohydrates were not moving to the ovary, their levels did decrease in the petals. This may be as a result of utilization in the system. The respiration rate of acetaldehyde-treated flowers has not be recorded. Fruit treated with acetaldehyde still respired, although at reduced rates (SALTVEIT and MENCARELLI, 1988; PESIS and MARINANSKY, 1992). It is possible that the decrease in carbohydrates recorded in the petals of acetaldehyde-treated carnations was as a result of their utilization to facilitate respiration, and other cellular functions.

The ovaries of acetaldehyde-treated flowers did not increase in dry weight as those of the control flowers did. In fact the dry matter levels of treated ovaries decreased. The moisture content was not effected. As they did not gain dry weight, it was clear that the ovaries were no longer acting as carbohydrate sinks. When ^{14}C sucrose was applied to the petals of acetaldehyde-treated flowers, as was expected, no radioactivity was recovered in the ovary. The development of the chloroplasts was also inhibited by acetaldehyde treatment. The chlorophyll content of treated flowers was significantly reduced in the ovary. This resulted in a yellow appearance of treated ovaries. The prevention of this development may have been due to the degeneration of the chloroplast and membrane damage, as has been recorded in the chloroplasts of STS and ethanol-treated carnation flowers (BUTLER and SIMON, 1970; THIMANN, 1980; COOK and VAN STADEN, 1983; WOOLHOUSE, 1984; GEPSTEIN, 1988). The ovary would have no longer been capable of photosynthesis, adding to the deficit of carbohydrates within the system.

The protein content of the cells of the petals and ovary was significantly reduced as a result of acetaldehyde treatment. This was an indiscriminant loss of protein, as no particular size of protein was effected more than any other. This may be the cause of the inhibition of ethylene formation. The activity of ACC oxidase was severely reduced. ACC production within the system was also reduced, and this would also have been effected by the protein loss. Some cellular enzymes, such as ADH and PDC remained active (Chapter 4). This combined with the fact that there was some protein still present in the cells, as opposed to total protein loss, suggests that the metabolic activity of the cells was reduced, but not entirely halted by acetaldehyde treatment.

The physiological effects of acetaldehyde application were very similar to those of the ethanol treatment. The prevention of ethylene formation, as well as the subsequent loss of protein content, decreased chlorophyll levels and carbohydrate status were common to both post-harvest treatments. This indicates that the roles played by ethanol and acetaldehyde on the post-harvest physiology of cut carnation flowers are very closely linked to one another. Certain effects were more pronounced in acetaldehyde-treated flowers. The destruction of the ovary appeared to be more severe in the acetaldehyde-treated flowers than in ethanol-treated flowers. Acetaldehyde-treated flowers showed greater losses in dry matter, carbohydrate and chlorophyll content. Visually, they also appeared more severely damaged, they were often soft to the touch implying severe membrane damage. Unlike ethanol, acetaldehyde is only found naturally in plant material at extremely low concentrations, and usually only as an intermediate compound (GUSTAFSON,

1934). The negative effects of acetaldehyde on the ovary may be as a result of phytotoxic responses to unnaturally high levels of the compound.

CHAPTER 7

CONCLUSIONS

The sale of cut carnation flowers is a worldwide industry, worth huge amounts of money. This has resulted in a great deal of scientific research into possible methods of extending the life of these commercially import organs. Until recently silver thiosulphate had been used ubiquitously as the commercial post-harvest treatment. However, as silver is a heavy metal, trade restrictions and environmental concerns have lead to the search for a suitable replacement post-harvest treatment.

Ethanol successfully extends the vase life of cut carnation flowers as effectively as silver treatment. This is achieved by the prevention of ethylene formation, chloroplast development, destruction of the carbohydrate relocation within the flowers and mass proteolysis within the cells. The result is that flowers almost cease to be metabolically active, and the bloom remains attractive for an extended period compared to normal flowers. Unfortunately however, ethanol would not be suitable for commercial use, as it must be applied as a continuous treatment. It also did not result in consistent longevity increases and is not effective against external ethylene sources.

During the course of this study it was discovered that the natural precursor of ethanol, acetaldehyde, also extended the vase life of cut carnation flowers. The physiological response of flowers to acetaldehyde treatment is almost identical to the response to ethanol. Ethylene formation, carbohydrate status, protein and

chlorophyll content are all inhibited or destroyed by acetaldehyde application. Unfortunately, also like ethanol, acetaldehyde does not prevent the action of exogenous ethylene, and if the treatment is stopped at any point, maximum longevity is not achieved. The longevity increase as a result of acetaldehyde application was not consistent, possibly due to variation in the age of flowers at harvest. These factors meant that acetaldehyde is also not a suitable candidate for a commercial post-harvest treatment.

The large number of similarities between ethanol and acetaldehyde application is the result of a strong alliance between the two compounds within cut carnation flowers. When ethanol is applied as a post-harvest treatment, and, if the conversion to acetaldehyde is blocked, no longevity increase is achieved. Likewise, if acetaldehyde is prevented from being converted to ethanol, carnation senescence is advanced. The activity of alcohol dehydrogenase in flowers treated with either compound is greater than in control flowers, even though there is a decrease in the protein content of the flowers as a result of treatment. The alcohol dehydrogenase is active in either orientation allowing the applied compound to shuttle between the alcohol and aldehyde forms easily. As a result, it is not possible to assign specific responses to specific compounds.

Although not a compound suitable for use as a commercial post-harvest treatment, research performed for this thesis has provided a deeper understanding of the physiological role of ethanol in senescing cut carnation flowers. It has also lead to the discovery of acetaldehyde as a compound capable of extending cut carnation flower longevity, and the complex interaction between applied ethanol and

acetaldehyde in post-harvest systems. This information can also be used to try and gain a deeper insight into the post-harvest role of both compounds in ripening tomatoes and other fruits, with the possibility of commercial benefits in this industry.

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APPENDIX A

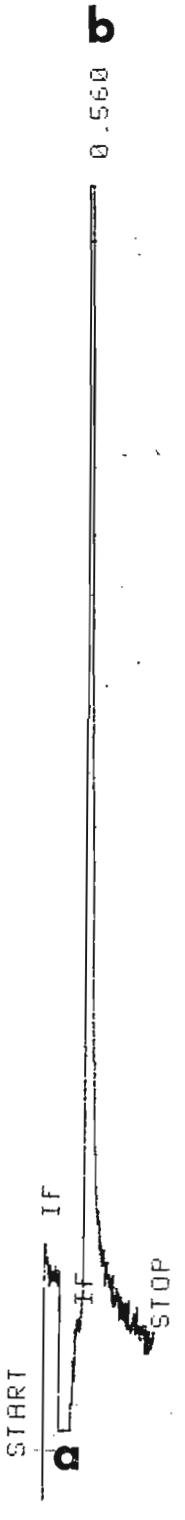

Figure A. Representative chromatogram for ethylene detection. Peak A = solvent front, B = ethylene.

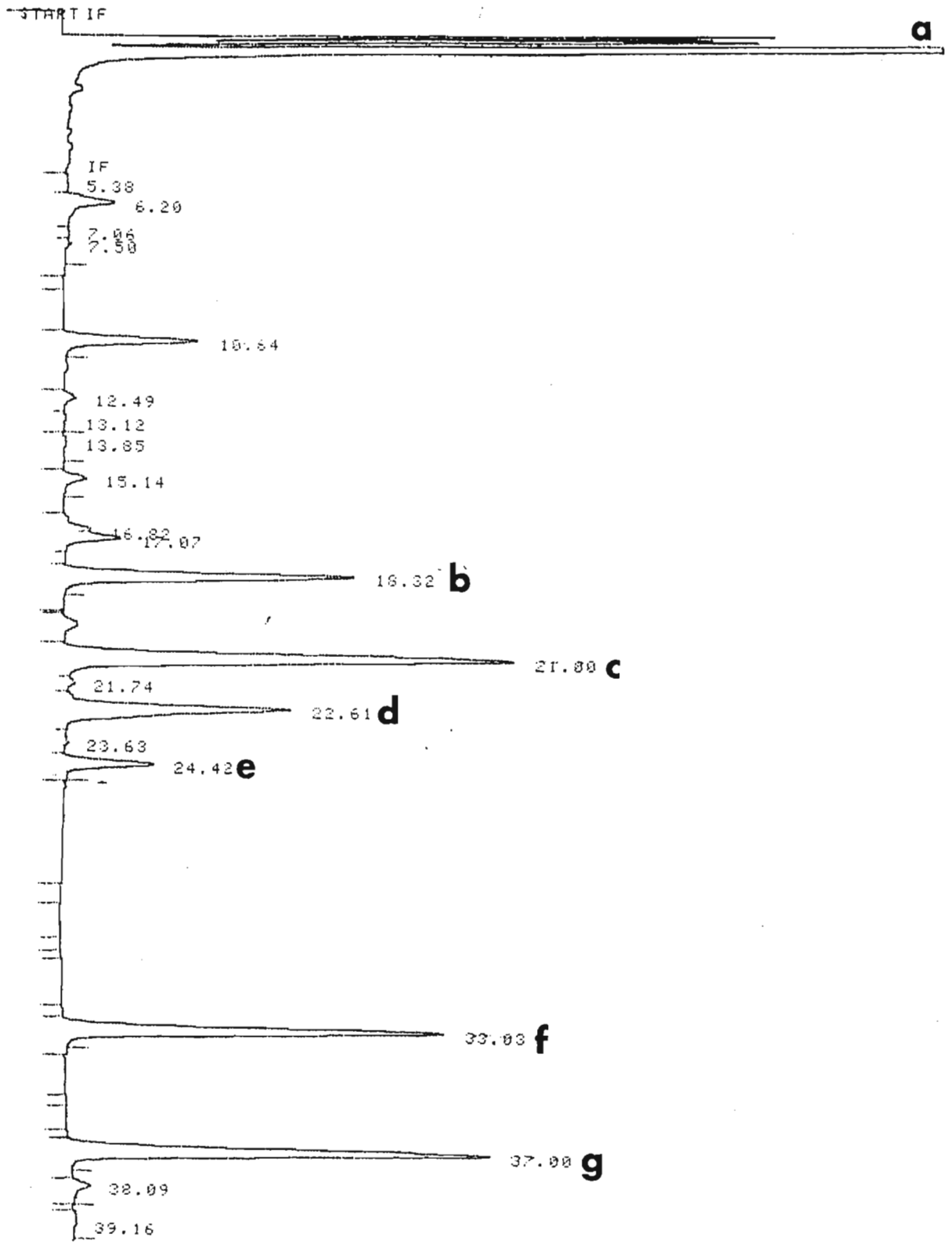

Figure B. Representative chromatogram for sugar detection with Sil A. Peak A = solvent front, B = xylose, C = sorbitol, D = fructose, E = glucose, F = phenyl-ß-D-glycoside standard and G = sucrose.

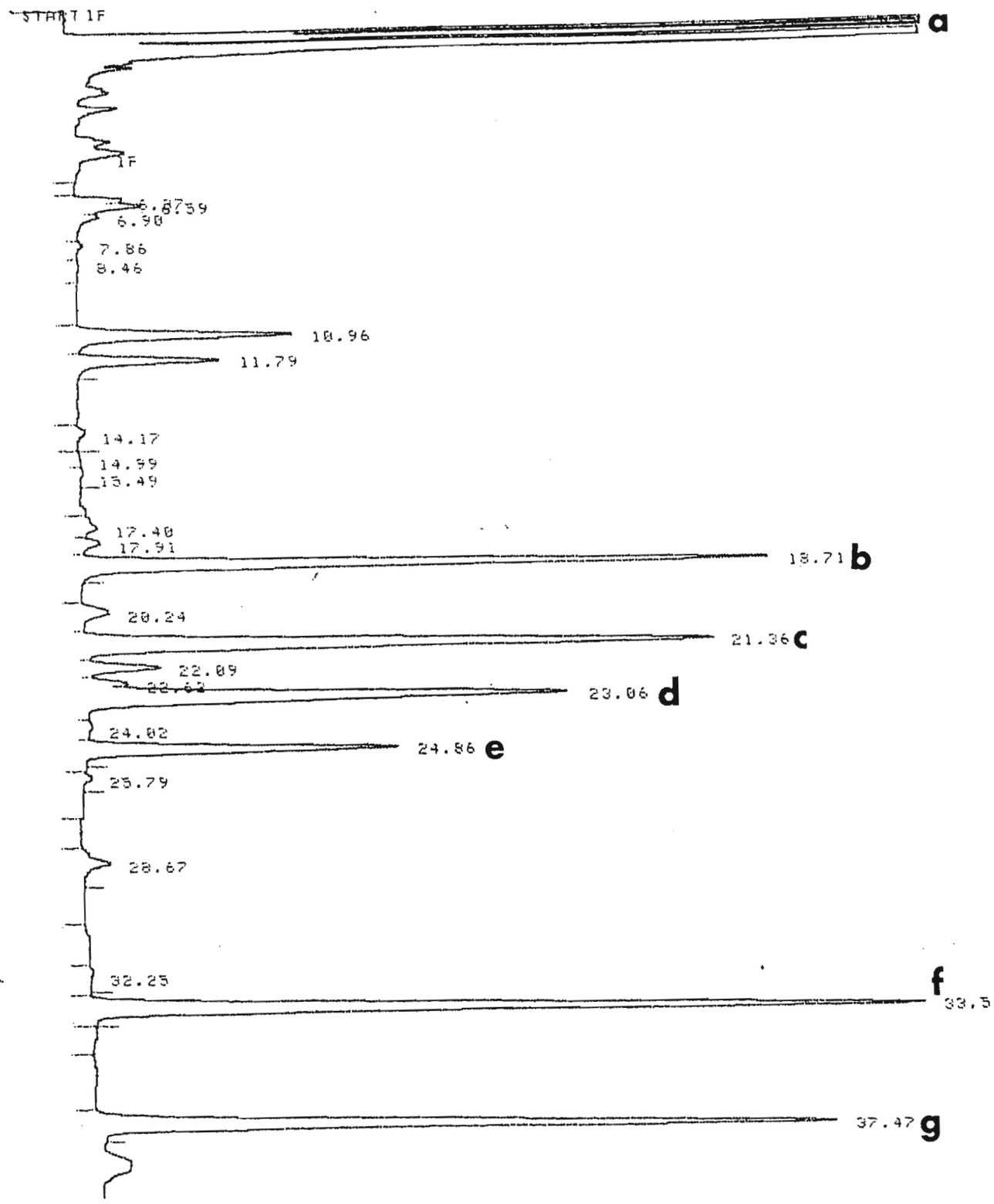

Figure C. Representative chromatogram for sugar detection with Sylon BT. Peak A = solvent front, B = xylose, C = sorbitol, D = fructose, E = glucose, F = phenyl-ß-D-glycoside standard and G = sucrose.

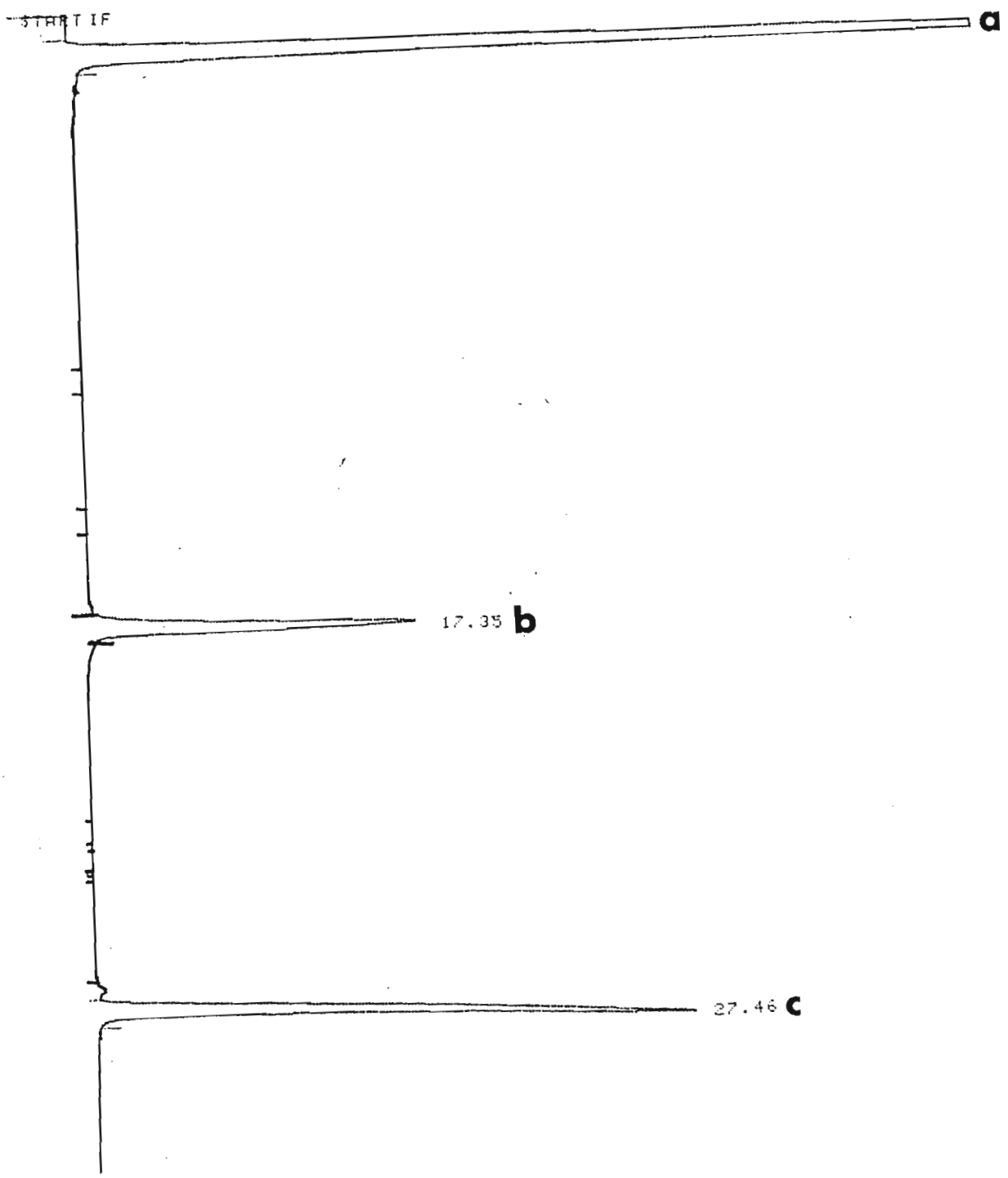

Figure D. Representative chromatogram for ethanol and acetaldehyde detection. Peak A = solvent front, B = acetaldehyde and C = ethanol.

APPENDIX B

Figure E. Entire SDS-PAGE gel for the second day of experimentation.

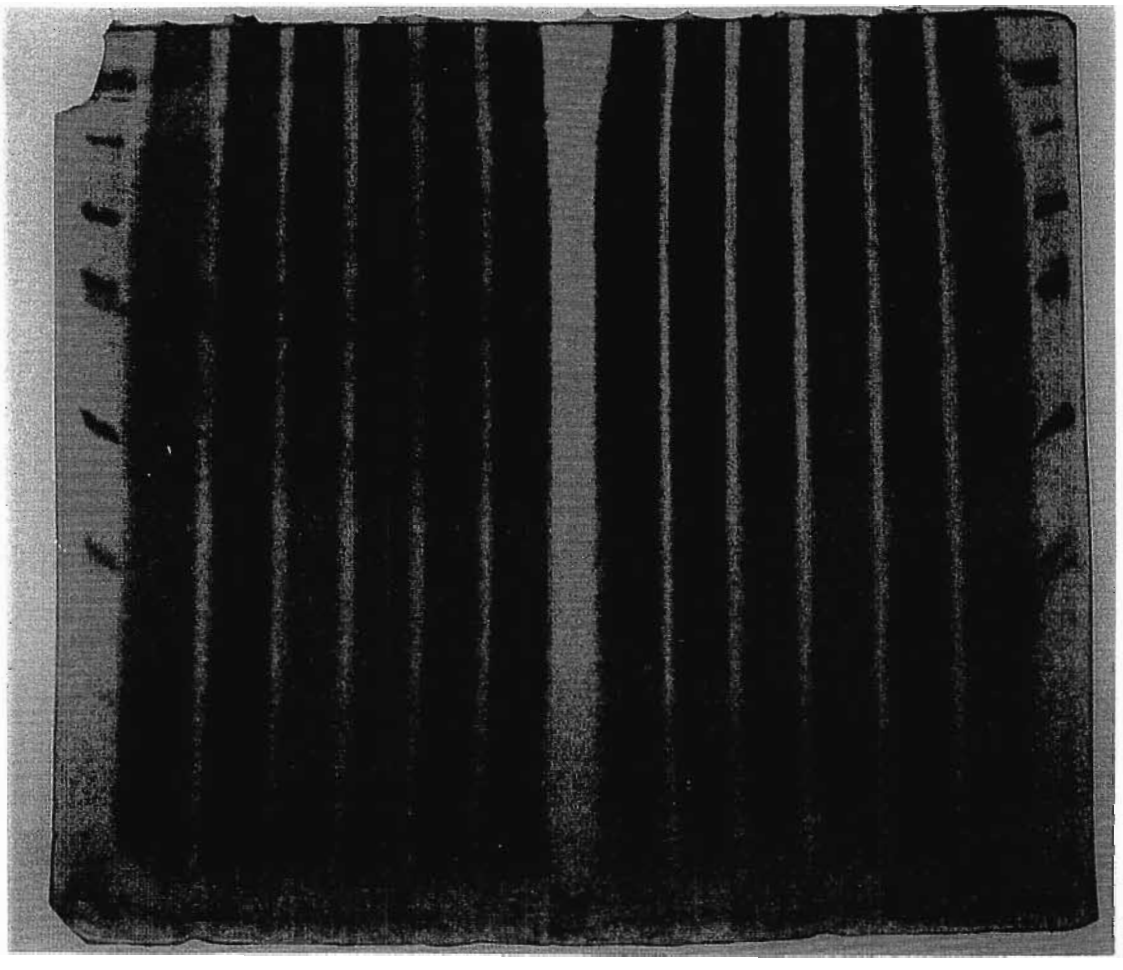

Figure F. Entire SDS-PAGE gel for the fifth day of experimentation.

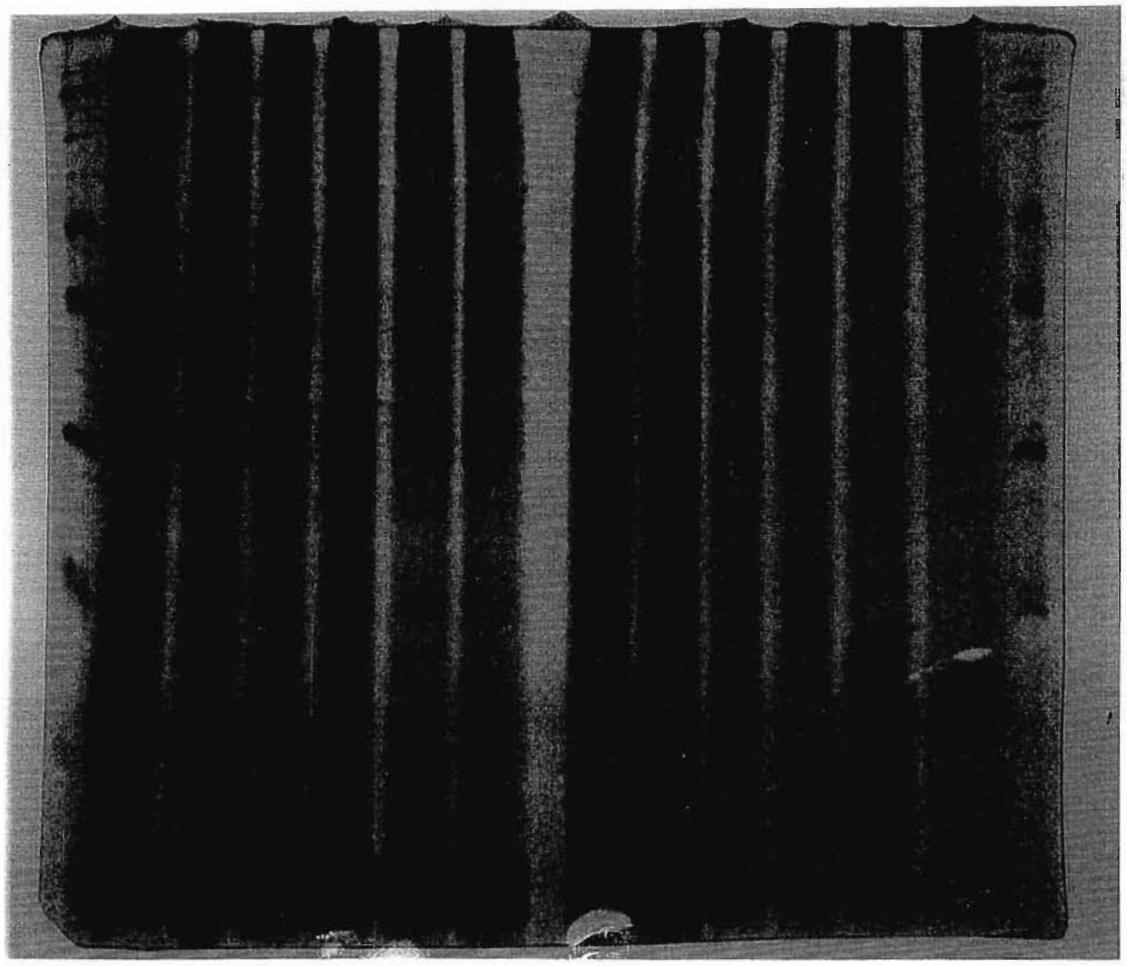

Figure G: Entire SDS-PAGE gel for the tenth day of experimentation.