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A biochemical analysis of the factors influencing the onset of senescence in ageing Dianthus caryophyllus L. flower parts.

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A BIOCHEMICAL ANALYSIS OF THE FACTORS INFLUENCING THE ONSET OF SENESCENCE IN AGEING *DIANTHUS CARYOPHYLLUS* L. FLOWER PARTS

A Dissertation Presented

by

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ABSTRACT

A BIOCHEMICAL ANALYSIS OF THE FACTORS INFLUENCING THE ONSET OF SENESCENCE IN AGEING DIANTHUS CARYOPHYLLUS L. FLOWER PARTS

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Senescence in ageing carnation flowers (Dianthus caryophyllus L. cv. Scanea 3C) is characterized by a climacteric rise of ethylene and an irreversible wilting in the petals. This investigation was initiated to elucidate the biochemical changes occurring subsequent to the onset of senescence in carnation flower parts.

Carnation flowers were harvested and kept in a controlled atmosphere for 0 to 9 days. On each sampling day, flower parts were excised, and different parameters of senescence were investigated. All parts produced synchronous respiratory and ethylene climacteric peaks seven days after opening. Peaks of 1-aminocyclopropane-1-carboxylic acid (ACC) activity, were detected at either six or seven days, after flower opening. Production of 1(malonylamino)cyclopropane-1-carboxylic acid (MACC), was synchronous in all ageing flower parts, with a large peak of activity observed at day three. This was followed by a 70 percent reduction of conjugate by day four. Another large peak was detected at seven days.

Exogenous MACC was applied to excised preclimacteric petals and ethylene production was monitored. MACC-induced ethylene and subsequent petal wilting were detected after approximately 33 hours. Radiolabelled ACC was administered to preclimacteric carnation flower petals and the

radioactive metabolites were analyzed. After incubating 24 hours, less than two percent of the label was recovered as ethylene. Another 20 and 10 percent were retrieved as nonmetabolized and conjugated ACC, respectively. Approximately six percent of the label was recovered as anthocyanins. The remaining metabolites were not characterized.

Levels of abscisic acid (ABA) were measured using an enzyme-linked immunosorbent assay (ELISA). ABA increased in the petals and in the green tissue six days after flower opening. An increase of ABA was detected in the receptacle after seven days. An increase of ABA in both the styles and the ovary occurred in the preclimacteric flower.

TABLE OF CONTENTS

APPENDICES

LIST OF TABLES

LIST OF FIGURES

C H A P T E R I

INTRODUCTION

Flower Senescence

Flowering is a unique developmental process. The initiation of a flowering structure from a vegetative bud is a pivotal event in the ontogeny of the plant. With flower initiation begins flower development and the sequence of events that will ultimately lead to senescence. The ephemeral nature of the flowering structure makes it a particularly attractive system to use when studying plant senescence. Flowers are also very complex structures, with senescing and nonsenescing tissues adjacent to each. The various flower parts are initiated from the same flower bud, but evolve very dissimilarly. This diversity between flower parts involved in the same physiological event can be used in correlative studies on the nature of senescing and nonsenescing tissues.

The pattern of senescence differs from flower to flower. Typically, flower senescence is marked by an irreversible wilting and/or abscission in the flower parts (Mayak and Halevy, 1981). In many flowering structures pollination immediately precedes the onset of senescence. The response to pollination, or in some cases fertilization, may be a wilting of the corolla as in carnations (Nichols, 1977) and petunia (Petunia hybrida) (Whitehead, et al., 1984b), or petal abscission as with cyclamen (Cyclamen persicum) (Halevy, et al., 1984).

Many metabolic changes are occurring in the senescing flower. In general, there is an overall decrease of protein content associated with senescing tissue (Thimann, 1981). In conjuncture with proteolysis, however, de novo synthesis of hydrolytic enzymes in senescing flower

petals has been reported (Mayak and Halevy, 1981). In senescing morning glory (Ipomea tricolor) petals, Baumgartner, et al. (1975) reported de novo synthesis of ribonuclease coincident with the first visible sign of petal wilting. In addition , when protein synthesis inhibitors were administered to senescing carnation flower petals, an inhibition of ethylene-induced senescence resulted, indicating a role for protein synthesis during senescence (Wulster, et al., 1982). Matile and Winkenbach (1971), studying the corolla of Ipomoea purpurea flowers, detected an increase in both RNase and DNAse after flower opening and just prior to petal fading. Concurrently with the increase of degradative enzymes was a decrease in total protein. They ascribed petal senescence coincident with the increase of degradative enzymes to autophagic activity in the vacuole. Studying electron micrographs of senescing petal cells, they observed invaginations of the tonoplast and shrinkage of the vacuole. Cell lysis was observed after eventual breakdown of the tonoplast. Burger, et al. (1986) detected ultrastructural changes in the tonoplast in ageing carnation flower petal cells prior to the increase of ethylene. They suggested that the degradative enzymes promoting the onset of senescence are located here and are attributed to the initiation of the degradation of membrane integrity. Butcher, et al. (1977) detected increases in degradative enzymes in both the vacuolar and cytoplasmic fractions of senescing flower petals. The vacuole is involved in storing secondary products such as plant pigments (Goodwin and Mercer, 1983). It is reasonable to suspect that this organelle is intricately involved in the partitioning and degradation of cellular constituents during senescence.

Flower senescence is also marked by drastic changes in pigmentation. Color changes are sometimes the first visible evidence that senescence has initiated. Petunia petals first undergo a change in pigmentation before they begin irreversible wilting (Whitehead, et al., 1984b). Conspicuous pigment changes are a common phenomenon in many different ageing tissue, notably those associated with fruit ripening and leaf senescence of deciduous trees. It is interesting to note the ecological importance ascribed to flower pigment changes occurring coincident with the onset of senescence. There can either be an accumulation of pigments, or a turnover of existing pigments with increasing petal age (Halevy and Mayak, 1979). In the former case, accumulation represents the shunting of metabolites to the formation of a secondary product in the tissue. When pigments are degraded the large pool of metabolites is mobilized to the rest of the flower. In some flowers, such as carnations, it appears that both accumulation and turnover of pigments are interrelated in the senescing petal. A sharp increase in anthocyanins can be detected coincident with a surge of ethylene production at the onset of senescence (Hanley, et al., 1988). Following the ethylene increase and the pigment accumulation comes degradation and a concomitant enlargement of the ovary (Trippi and Paulin, 1984).

Carotenoid changes in flower petals are very similar to those found in ripening fruit (Goldschmidt, 1981). Simpson, et al. (1975) followed the changes of petal plastids in developing Strelitzia reginae flowers. They observed typical ultrastructural changes including chloroplasts evolving into chromoplasts. They also observed an increase in the total concentration of carotenoids with increasing petal age. An accumulation

of carotenoids is not always a prerequisite for senescing or ripening tissue, however (Mayak and Halevy, 1981).

Patterns of anthocyanin production associated with senescence differ from flower to flower (Leshem, et al.,1986). In some senescing flowers a decolorization takes place as the flower ages and senesces. In the chicory flower this has been attributed to enzymatic activity similar to catecholase, which increased with increasing flower age (Proctor and Creasy, 1969). The opposite coloration pattern occurs in Hibiscus mutabilis, which starts out colorless and accumulates large quantities of anthocyanins by the end of the day just prior to wilting (Lesham, et al., 1986). The ephemeral nature of hibiscus flowers is associated with increased ethylene production concurrent with wilting (Woodson, et al., 1985). Although increased ethylene production does not appear to be related to the early accumulation of anthocyanins in developing hibiscus flowers, its increase coincident with the onset of senescence might be coordinating the mobilization and/or metabolism of pigments or their mobilization from the wilting petal to the rest of the flower.

Pollination causes an acceleration of the onset of flower senescence in many species (Mayak and Halevy, 1981). Frequently the same pattern of senescence is observed in both pollinated and unpollinated flowers, but senescence of the latter is initiated much later. Unpollinated, fully open carnation flowers begin to senescence approximately five to seven days after harvest (Bufler, et al., 1980, Hanley, et al., 1988), while wilting in pollinated flowers commences after about two to three days (Nichols, 1977). In Petunia, either pollination or mechanical wounding resulted in accelerated petal wilting 4 to 6 days prior to the untreated

control flowers (Hoekstra and Weges, 1986). In many plant species the onset of senescence in both pollinated and unpollinated flowers is associated with increased production of ethylene (Halevy and Mayak, 1981, Nichols, 1984). In cyclamen flowers, though, increased ethylene and corolla abscission was observed only after flowers were pollinated (Halevy, et al., 1984). The unpollinated control flowers senesced much slower and without abscission of any flower parts. There has been some speculation as to the mechanism by which pollination stimulates ethylene production and senescence. Early studies postulated that the trigger for ethylene production might be coming from a substance found in the pollen (Mayak and Halevy, 1981). Burg and Dijkman (1967), working with Vanda orchids, proposed that auxin detected in the pollen was being transported throughout the flower upon pollination, mediating ethylene-induced flower senescence. While auxin does stimulate ethylene production (Imaseki, 1986), it has also been found to be very immobile in stylar tissue (Strauss and Arditti), and has been regarded as an unlikely candidate for the trigger of ethylene-induced senescence (Hoekstra and Weges, 1986). The ethylene precursor, 1-amino-cyclopropane-1-carboxylic acid (ACC), has been detected in carnations (Whitehead, et al., 1983) and petunia (Whitehead, et al., 1984b) pollen grains and was suggested to be the mobil substance triggering flower senescence upon pollination (Reid, et al., 1984, Whitehead, et al., 1983). However, substantial ethylene-forming enzyme (EFE) activity has been detected in the stylar tissue of both carnations (Manning, 1981) and petunia flowers (Hoekstra and Weges, 1986). This enzyme is responsible for the oxidation of ACC to ethylene and its activity in the styles would impede the movement of the ethylene

precursor to the rest of the flower. Hoekstra and Weges (1986) detected a small amount of ethylene production from petunia styles approximately 10 minutes after pollination. They inhibited this ethylene production by applying aminoethoxyvinylglycine (AVG), an inhibitor of ACC synthesis, and concluded that the ethylene must be emanating from newly synthesized ACC and not ACC present in the pollen grains. In order for their conclusion to be correct, the ACC synthase enzyme must be active at this time, due to the fact that protein synthesis to form ACC synthase would not occur in 10 minutes. They concluded that pollination-induced senescence is derived from some component of the physical wounding of the stylar tissue upon pollination. It is possible that the wounding somehow predisposes the tissue for the onset of ethylene-induced senescence.

Study Flower: Dianthus caryophyllus L. cv. Scanea 3C

The carnation (Dianthus caryophyllus L.) is a member of the Caryophyllaceae family. It is a perennial plant with branching stems and glaucous leaves formed in opposite and decussate pairs (Bunt and Cockshull, 1986). The cultivar Scanea 3C is a red, standard carnation formed from the terminal bud. During development lateral shoots and buds are removed, leaving a more vigorous apical bud. For cultivars of miniature carnations the terminal bud is removed to stimulate the formation of smaller lateral buds. The flower is comprised of approximately 40 to 60 petals enclosed in a five membered-calyx adjoined by two pairs of bracts. The superior ovary generally has two to three carpels with no distinct stigma but rather a stigmatic surface on the style. Most standard carnations do not have stamens, but instead,

rudimentary structures similar to staminoids develop (Bunt and Cockshull, 1986). Many miniature carnation cultivars do have stamens, which can serve as a source of viable pollen in pollination experiments.

Carnation flowers have a very distinct ageing process, making them an excellent plant to use when studying flower senescence. Most of studies detailing senescence in carnation flowers have been done using cut flowers. It is assumed that phenomena exhibited in cut flowers are similar to those in attached flowers.

The first visible sign of flower senescence in carnations is an irreversible wilting in the petals. This wilting is coincident with a surge of ethylene (Nichols, 1971), similar to the ethylene climacteric in ripening fruit (Rhodes, 1981). It has been proposed that some stimulus produced in one part of the flower moves into the petals, inducing the ethylene surge and the onset of senescence (Bufler, et al., 1980, Sacalis, et al., 1983). However, when the ovary and/or the styles were removed prior to the onset of senescence, there was no effect on time to wilting in the petals (Mor, et al., 1980, Sacalis and Suk, 1987). In addition, detached petals ageing independently senesced similarly to petals still attached to the intact flower (Mor and Reid, 1980). This response was age-dependent, in that very young, detached petals took longer to senesce than attached petals (Mor, et al., 1980). Nichols (1977) measured the ethylene production in ageing carnation flowers with the petals or the gynoecium excised. For the first five days neither flower produced any significant amount of ethylene, but by six days both were producing ethylene. He concluded that ethylene evolution in the petals and the gynoecium were independent of each other.

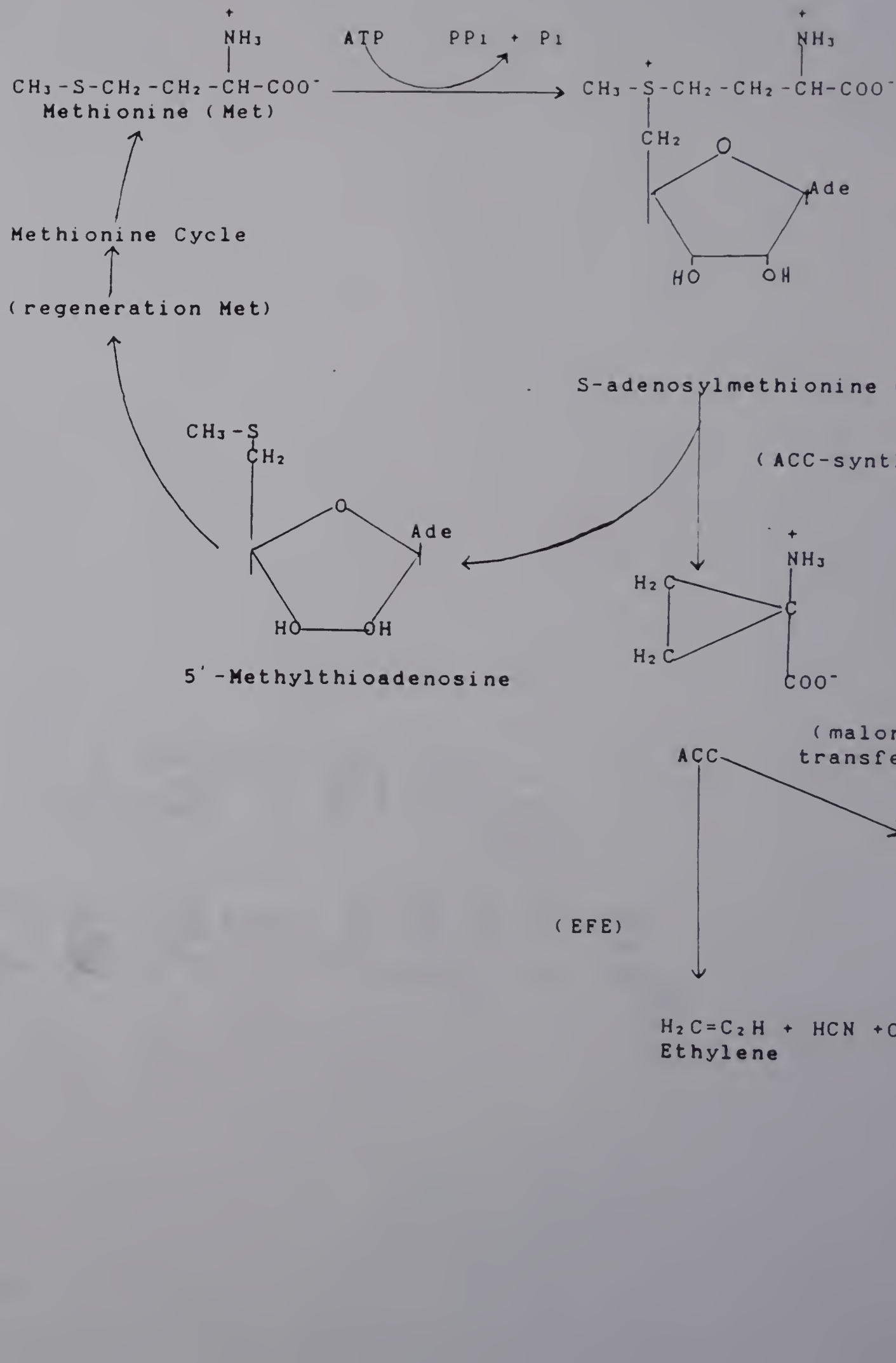
The ethylene-induced wilting response occurs similarly in both pollinated and unpollinated carnation flowers, except that it occurs sooner with pollination. In addition, analogous increases of the ethylene precursor, ACC, were detected in both pollinated (Nichols, et al., 1983, Reid, et al., 1984) and unpollinated flowers (Bufler, et al., 1980) just prior to the ethylene surge: at two days in the pollinated and at six days in the unpollinated flowers. Pollination might be acting to suppress the activity of an inhibitor in the unpollinated flower, or to stimulate some senescence inductor in the newly pollinated flower.

Ethylene and its Role in Carnation Flower Senescence

The biochemical pathway of ethylene synthesis was first elucidated in ripening apple fruit (Adams and Yang, 1979, Lieberman and Mapson, 1967). The synthesis of ethylene is derived from the amino acid methionine (MET) and continues through the formation of the intermediates s-adenosyl-methionine (SAM) and ACC (Figure 1). When ripening tissue was reated with aminoethoxyvinylglycine (AVG), a known inhibitor of pyridoxal phosphate-mediated enzyme reactions, the conversion of SAM to ACC was inhibited, but AVG did not affect the metabolism of methionine to SAM, or the conversion of exogenous ACC to ethylene (Adams and Yang, 1979). This work led to the isolation of ACC-synthase, the enzyme catalyzing the conversion of SAM to ACC (Boller, et al., 1979, Yu, et al., 1979). The conversion of ACC to ethylene is mediated by the ethylene-forming enzyme (EFE) (Yang, 1985). In the reaction mechanism put forth by Peiser, et al. (1984), the oxidation of ACC results in equimolar concentrations of

ethylene, CO_2, and HCN (Figure 1). Although EFE has never been isolated, studies have shown it to be oxygen-dependent, heat-denaturable, inhibited by free radical scavengers, and requiring an intact membrane for activity (Yang, 1985). The continuous supply of methionine needed during periods of high ethylene evolution is accounted for by a cyclic generation of the amino acid, which is driven by the addition of ATP (Yang, 1985). In some plant tissues ACC is not metabolized into ethylene, but instead is conjugated to form 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC) (Figure 1) (Hoffman, et al., 1982). This metabolite is generally regarded as a poor ethylene precursor and relatively inactive (Hoffman, et al., 1983a). Some plant tissues, though, have the enzymatic ability to hydrolyze MACC back to ACC and ethylene (Jiao, et al., 1986). The activity of both EFE and the malonyltransferase enzyme may be an important site of regulation, as they are competing for the same substrate, free ACC (Kionka and Amrhein, 1984). Recent studies have reported on the relationship between the malonylation of D-amino acids and ACC (Liu, et al., 1983, Matern, et al., 1984, Kionka and Amrhein, 1984, Su, et al., 1985). These amino acids, notably D-phenylalanine, competitively inhibit the formation of MACC resulting in free ACC going to ethylene formation. Ethylene production was stimulated in cocklebur (Xanthium pennsylvanicum) seeds after presoaking in different D-amino acids; with D-methionine and D-phenylalanine being the most effective (Satoh and Esashi, 1981). Su, et al.(1985) presented evidence that the malonylation of both D-amino acids and ACC is mediated by the same enzyme. Further research is needed to clarify the mechanism involved in

Figure 1. The ethylene biosynthetic pathway (Yang, 1985).


NH3
ATP
PP1 + P1
NH3
CH3-S-CH2-CH2-CH-COO-
Methionine (Met)
CH3-S-CH2-CH2-CH-COO-
CH2
O
Ade
HO
OH
Methionine Cycle
(regeneration Met)
S-adenosylmethionine (SAM)
(ACC-synthase)
CH3-S
CH2
O
Ade
HO
OH
5'-Methylthioadenosine
NH3
H2C
C
H2C
COO-
(malonyl
transferase)
ACC
MACC
(EFE)
H2C=C2H + HCN +CO2
Ethylene

regulating the levels of free ACC in plant tissue. In many ethylene generating tissues the limiting step in the pathway is the conversion of SAM to ACC (Yang, 1985). In preclimacteric fruits and flowers, though, this is generally not the case, and the point of regulation appears to be at the step from ACC to ethylene (Yang, 1981). Exogenous application of ACC to excised preclimacteric carnation petals (Manning, 1981), resulted in a lag period before an increase of ethylene was detected, indicating that the substrate was inducing a response. This reaction was concentration-dependent; increasing ACC concentration resulted in a decreased lag time to the start of ethylene production. When EFE activity was evaluated in premature carnation flower parts beginning in the closed bud stage, only the styles had any significant enzymatic activity (Manning, 1981). Even before petal emergence, the premature styles still contained significant ethylene-forming enzyme activity. The high activity of EFE in the developing styles may play a role in pollination-induced accelerated senescence in these flowers.

Ethylene is proposed to mediate its effect on senescing carnation flower petals by altering membrane composition and viscosity (Thompson, et al., 1982), which ultimately leads to leakiness and loss of turgor (Mayak, et al., 1977). Similar membrane changes have been observed in other senescing flower tissue (Suttle and Kende, 1980). The increased production of ethylene at the onset of flower senescence appears to act as an accelerator, inducing damage to the cell membrane (Mayak, et al., 1977, Thompson, et al., 1982, Eze, et al., 1986). When ethylene inhibitors were applied to carnation flowers prior to the ethylene surge, increased membrane microviscosity in the petals was still detected, but

very late in the vase life of the cut flower (Thompson, et al., 1982).

The Influence of Abscisic Acid in Senescing Carnation Flowers

The endogenous level of abscisic acid (ABA) increases in ageing flower tissue, and has been regarded as a regulator in flower senescence (Mayak and Halevy, 1981). Although it seems to be intimately involved in the senescence of carnation flowers (Ronen and Mayak, 1981, Nowak and Veen, 1982, Eze, et al., 1986), a specific mode of action has not been clearly defined. ABA, analogous to ethylene, is generally considered to be an inhibitor of plant growth and development (Hirai, 1986). An increase of endogenous ABA is frequently coupled to an increase in degradative enzymes (Ho, 1983), and this may be a possible mechanism for ABA action in senescing tissue. Abscisic acid also has an effect on membrane permeability, most notably in regard to the induction of K^+ influx and H^+ efflux regulating stomatal closure during periods of water stress (Davies and Mansfield, 1983). ABA is known to cause increased membrane permeability (Van Steveninck and Van Steveninck, 1983), and may be acting similar to an ionophore to form channels in the membrane (Lea and Collins, 1979). Brengle, et al. (1988), using artificial lipid bilayer vesicles, observed ABA-induced membrane instability which was dependent upon the concentration of ABA, the phospholipid components (phosphatidylcholine alone was not disrupted), and pH of the surrounding medium. ABA may be acting in concert with ethylene to mediate its effect on loss of membrane integrity and irreversible wilting in carnation flower senescence.

Studies have tried to correlate the increases of ethylene and ABA in sencescing flower tissue. In excised rose petals an increase of endogenous ABA was detected subsequent to the increase of ethylene (Mayak and Halevy, 1972). When these same flowers were treated with exogenous ABA, ethylene production was inhibited. It was proposed that ABA might be controlling ethylene production by a negative feedback mechanism. In contrast, exogenous application of 50 uM ABA to intact carnation flowers shortened flower longevity by hastening ethylene production (Mayak and Dilley, 1976). When endogenous ABA was measured in ageing carnation flower parts, the increase was detected concomitant with or prior to the increase of ethylene (Nowak and Veen, 1982, Eze, et al., 1986). In order to help define the roles of ABA and ethylene in ageing carnation flowers, it is imperative to separate the activity of each hormone as they relate to a specific stage of development in the flower.

The objectives of this study were to determine what biochemical changes are occurring in the ageing carnation flower which might indicate a predisposition for the onset of senescence. In the subsequent chapters I present evidence in support of the hypothesis that changes are occurring subsequent to the onset of flower senescence which may be used as indicators influencing the production of ethylene and the onset of flower wilting.

C H A P T E R II

METABOLISM OF AGEING CARNATION FLOWER PARTS AND THE EFFECTS OF 1-(MALONYLAMINO)CYCLOPROPANE-1-CARBOXYLIC ACID-INDUCED ETHYLENE

Introduction

Petal wilting is a consequence of senescence in carnation flowers. Production of a burst of ethylene is coincident with this first visible sign of senescence (Nichols, 1971). The immediate signal responsible for the increased ethylene is not yet known. It has been proposed that a stimulus produced in one part of the flower moves into the petals causing the ethylene surge (Hsieh and Sacalis, 1986, Reid, et al., 1984), although removal of either the ovary or the styles does not seem to affect petal senescence (Mor, et al., 1980, Sacalis and Suk, 1987). It has been observed that detached petals senesce similarly to those still attached to the intact flower (Mor and Reid, 1980, Mor, et al., 1980).

1-aminocyclopropane-1-carboxylic acid (ACC) is the immediate precursor to ethylene biosynthesis in higher plant tissue (Adams and Yang, 1979). An increase of endogenous ACC levels precedes ethylene production in all parts of the flower (Bufler, et al., 1980). When petals detached from flowers producing considerable amounts of ethylene were treated with AOA, an inhibitor of ACC synthase, a marked decrease in ethylene production and a five-fold decrease in ACC concentration occurred (Mor and Reid, 1980). This suggested that the petals have the ability to synthesize their own ACC.

1-(malonylamino)cyclopropane-1-carboxylic acid (MACC) is well established as the primary conjugate form of ACC (Hoffman, et al., 1982). In some systems MACC is the inactive endproduct of ACC not being oxidized

to ethylene (Hoffman, et al., 1983a, Hoffman, et al., 1983b), the MACC then serving as a poor ethylene precursor (Hoffman, et al., 1983a). Significant quantities of MACC have been detected in certain tissues (Hoffman, et al., 1983a, Knee, 1985, Mansour, et al., 1986). Jiao, et al. (1986) demonstrated that exogenous application of MACC to various vegetative tissues could induce some ethylene production. They detected MACC-hydrolase activity in the cell-free extract of watercress stems. It was concluded that several hours of incubation in high concentrations of MACC were necessary to induce the enzyme and maintain its continued activity. The present study investigated ethylene and CO_2 production, along with concentrations of ACC and MACC, during ageing of different carnation flower parts; these parts differ in longevity, i.e., petals, styles, and ovary senesce prior to the receptacle and green tissue. It was our intent to determine whether or not all parts behaved in synch or if one or more flower part(s) acted singularly to possibly mobilize the ethylene precursor(s), triggering senescence. It was also the intent of this research to determine whether or not exogenous MACC could be utilized as an effective ethylene precursor in carnation, resulting in ethylene-mediated senescence, after we observed marked reductions in MACC concentrations in the tissues.

Materials and Methods

Plant Material

Carnation flowers (Dianthus caryophyllus L. cv. Scanea 3C) were grown under normal greenhouse conditions. An accurate assessment of flower development was achieved by tagging flowers just prior to flower opening. A primilary study determined days to flower opening after following bud

length of closed carnation buds to first fully open flowers (appendix, Fig. 16). Flowers were then harvested when outer petals first reflexed at right angles to the stem. Stems were cut to 30 cm and placed individually in 50 ml test tubes containing 200 mg l^{-1} Physan™ (a microbicide) solution. Flowering stems were kept in a growth chamber at 22 C under continuous fluorescent lighting for 0 to 9 d. On each sampling day 15 flowers were divided into styles, ovary, petals (five from outer whorl), receptacle, and green tissue (calyx, bracts, sepals, two leaves, and 12 cm of stem) to be used for different analyses. These experiments were replicated four times, a replicate being a repeat of the entire experiment, conducted at different times of the year. For MACC metabolism studies petals were removed from the outermost whorl of flowers 1 d after opening. All excised petal experiments were replicated three times.

Chemicals

All solvents were of analytical grade and purchased commercially. ACC was purchased from Sigma. [2,3-^{14}C] ACC (80 uCi/uM) was from Research Products International (Mount Prospect, Illinois). MACC and AVG were generous gifts of Drs. S.F. Yang and A.K. Mattoo, respectively.

Determination of Ethylene and CO_2

At each sampling day individual floral parts were weighed and placed in 20 ml vials (styles, ovary, receptacles) or 25 and 125 ml flasks (petals and green tissue) which were sealed with rubber septa. All parts were incubated for 1 h in the light. A similar experiment was conducted in the dark and results were not significantly different (appendix, Figs. 17 and 18). After the incubation period, ethylene was

determined from a 1 ml gas sample withdrawn from the headspace using a 3 ml syringe. This was injected into a Shimadzu GC-8A gas chromatograph equipped with an activated alumina column and flame ionization detector. Data were compiled using a Shimadzu GC integrator. Immediately following, a 2 ml gas sample was withdrawn and injected into a Varian GC equipped with thermal conductivity detector to assay for CO_2.

ACC and MACC Extraction and Determination

After gas analyses, plant parts were frozen and lyophilized. Dried petals and green tissues were ground in a Wiley mill (20 mesh screen). The remaining plant parts were ground to a fine powder with a mortar and pestle. Tissue samples of 200 mg were extracted in 10 ml 80% (v/v) ethanol at 4 C for 24 h. Samples were centrifuged at 5000x g and the pellet was resuspended in ethanol and recentrifuged. Ethanolic fractions were combined and evaporated under vacuum at 40 C and the residue was taken up in 5 ml of distilled H_2O. ACC was determined by a slightly modified version of Lizada and Yang (1979). A 0.25 ml aliquot from the aqueous extract was combined in a test tube with 0.1 ml of 10 mM $HgCl_2$ and 0.55 ml distilled H_2O. Each test tube was sealed with a rubber septum and 0.1 ml of commercial bleach (5% NaOCl) and saturated NaOH (2:1, v/v) was added with a 1.0 ml syringe. The test tube was vortexed 20 sec and placed in ice. After 5 min a 1 ml gas sample was withdrawn from the headspace of the tube and analyzed for ethylene. Authentic ACC was added as an internal standard to a replicate tube. Efficiency of conversion of ACC to ethylene was generally between 80 and 90 %. Conjugated ACC was hydrolyzed to ACC units following the method of Hoffman, et al. (1982) and analyzed as indicated above. Authentic MACC

was also added to a replicate tube to determine efficiency of the assay. The efficiency of conversion of MACC to ACC and then to ethylene was generally 75 %.

Identification of ACC and MACC Fractions

Labeled [2,3-^{14}C] ACC (40.0 KBq/10 ul) was added to crude petal extract and passed through a Dowex-50 (H+ form) ion exchange column. Radiolabeled ACC was eluted from the cationic column with 2 N NH_4OH. After elution and evaporation the basic fraction was chromatographed on a silica gel thin-layer chromatography plate. This was developed for 14 cm in butanol:acetic acid:water (4:1:1.5, v/v), air dried, and developed again (Lizada and Yang, 1979). After drying, the plate was divided into 10 equal R_f fractions which were scraped and eluted individually with 50 % (v/v) ethanol. After centrifugation and evaporation to aqueous, a 0.5 ml aliquot was taken from each R_f and combined with 10 ml scintillation solution (Scintiverse II, Fisher). Efficiency of the scintillation counter was approximately 85% . A similar petal extract, but with no labeled ACC, was put through a cationic exchange column to identify endogenous ACC, assayed as ethylene. In addition, the acid eluate from the H+ column was run through Dowex-1 (OH-form). The ACC conjugate was eluted from the column with 6 N formic acid (Hoffman, et al., 1982). Authentic MACC was co-chromatographed with the acidic fraction following the procedure outlined above. Activity was assayed as ethylene production after hydrolysis to ACC.

One petal per treatment was incubated on filter paper in 20 ml vials containing 1 ml feeding solution. After 24 h most of the solution had been absorbed. After addition of 0.5 ml distilled water, an aliquot was

taken to measure ACC and MACC uptake. All tissues were kept in continuous fluorescent light at 22 C. Incubation media included various concentrations and combinations of ACC (1.0 mM), MACC (0.5-1.0 mM), AVG (0.1 mM), $CoCl_2$ (0.1-1.0 mM), and 4 mM silver thiosulfate complex (STS) (Veen, 1979) as indicated. Control petals were treated with distilled water. To determine the effects of exogenous ACC and MACC, ethylene was measured every 4 h, starting at 24 h, until petals began inrolling, and on each subsequent day until control petals inrolled. In the MACC-plus-inhibitors experiment ethylene was analyzed every 3 h from 24 to 48 h. At each sampling time a 1 ml gas sample was withdrawn from the headspace and analyzed as stated above. After sampling, vials were uncapped, flushed with air and returned to the growth chamber. Vials were capped again 1 h prior to next sampling time.

Results

Production of Ethylene and Carbon Dioxide in Ageing Floral Parts.

The receptacle, petals, and green tissues did not start producing increased rates of ethylenc until at least 5 d. Both the ovary and the styles produced very small amounts of ethylene from 0 to 4 d, with increasing amounts starting at 5 d. All floral parts produced ethylene peaks 7 d after opening (Fig. 2). The styles produced the most ethylene (276 nl g^{-1} h^{-1}). The petals and ovaries both produced approximately 60 nl g^{-1} h^{-1} at the peak. The receptacle and green tissue produced less than one tenth of the total amount produced by either the petals or the ovaries at this time. Similar climacteric rises in respiration were observed in the individual parts, as measured by CO_2 production (Fig. 3).

Figure 2. Ethylene production of excised carnation flower parts from flowers harvested (cut) at 0 days and aged 0 to 9 days at 22 C. The bars represent SE of four replications.

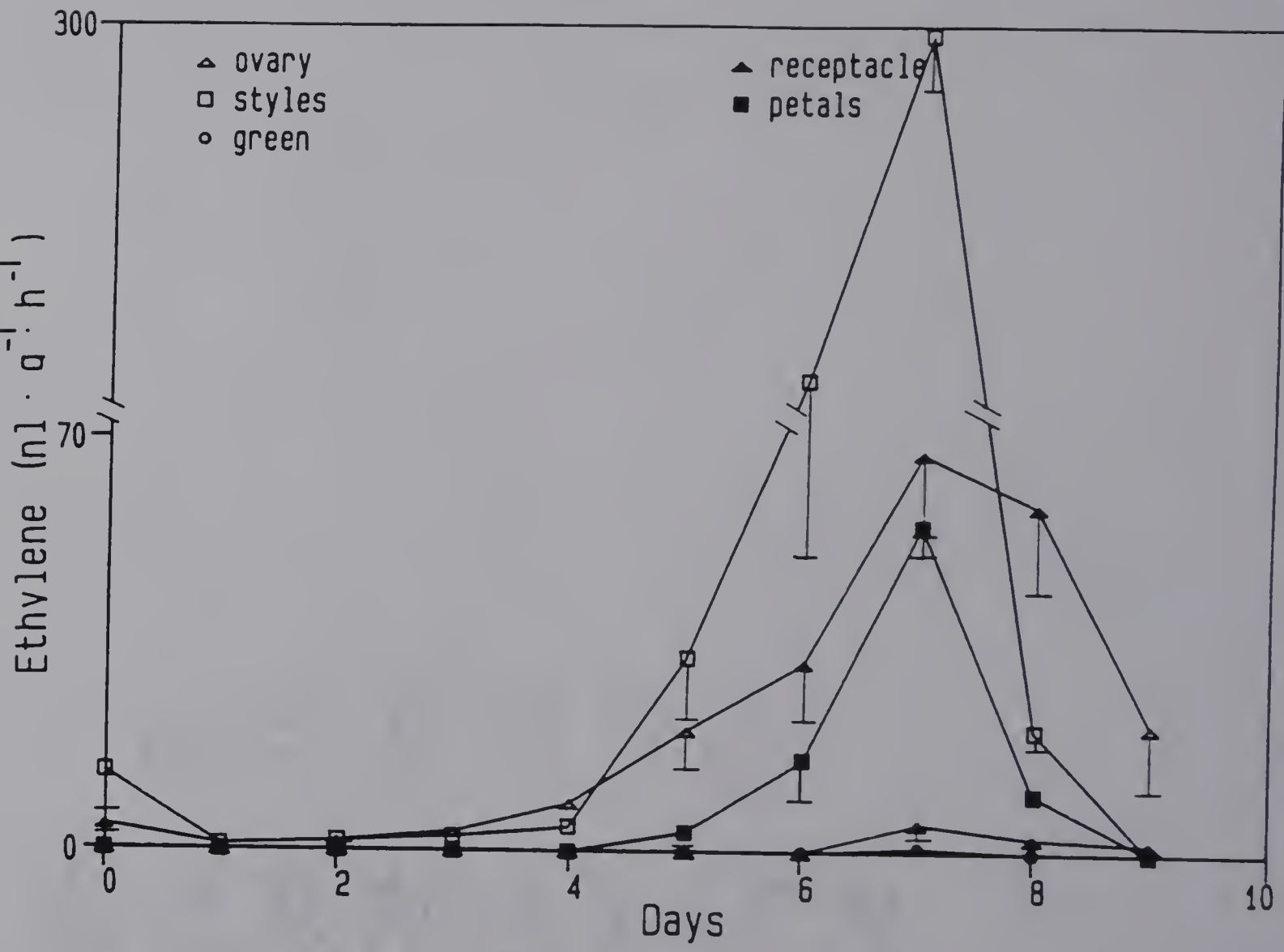
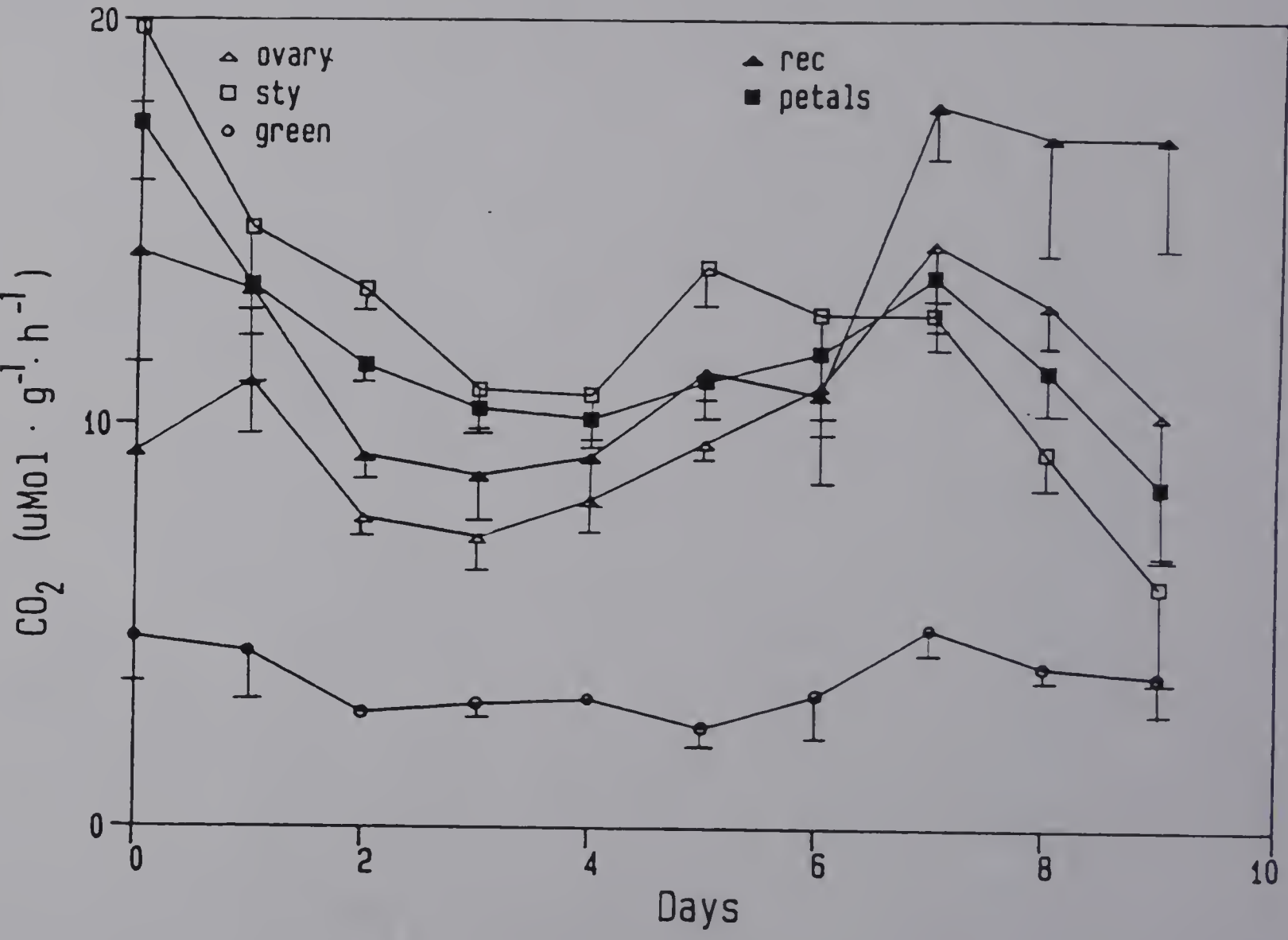

300
70
0
ovary
styles
green
receptacle
petals
Ethylene (nl · g⁻¹ · h⁻¹)
0
2
4
6
8
10
Days

Figure 3. Carbon dioxide production of excised carnation flower parts from flowers harvested (cut) at 0 days and aged 0 to 9 days at 22 C under continuous light. The bars represent SE of four replications.


20
10
0
ovary
sty
green
rec
petals
CO_2 (uMol · g^{-1} · h^{-1})
0
2
4
6
8
10
Days

Carbon dioxide levels peaked at 7 d except in styles, where respiration peaked at 5 d and remained high until 7 d. The receptacle kept producing large amounts of CO_2 even after the climacteric peak, whereas in the other parts it declined sharply. The green tissue produced significantly less CO_2 than the rest of the flower parts throughout the experiment, whether the experiment was run in light or dark (appendix, Fig. 18).

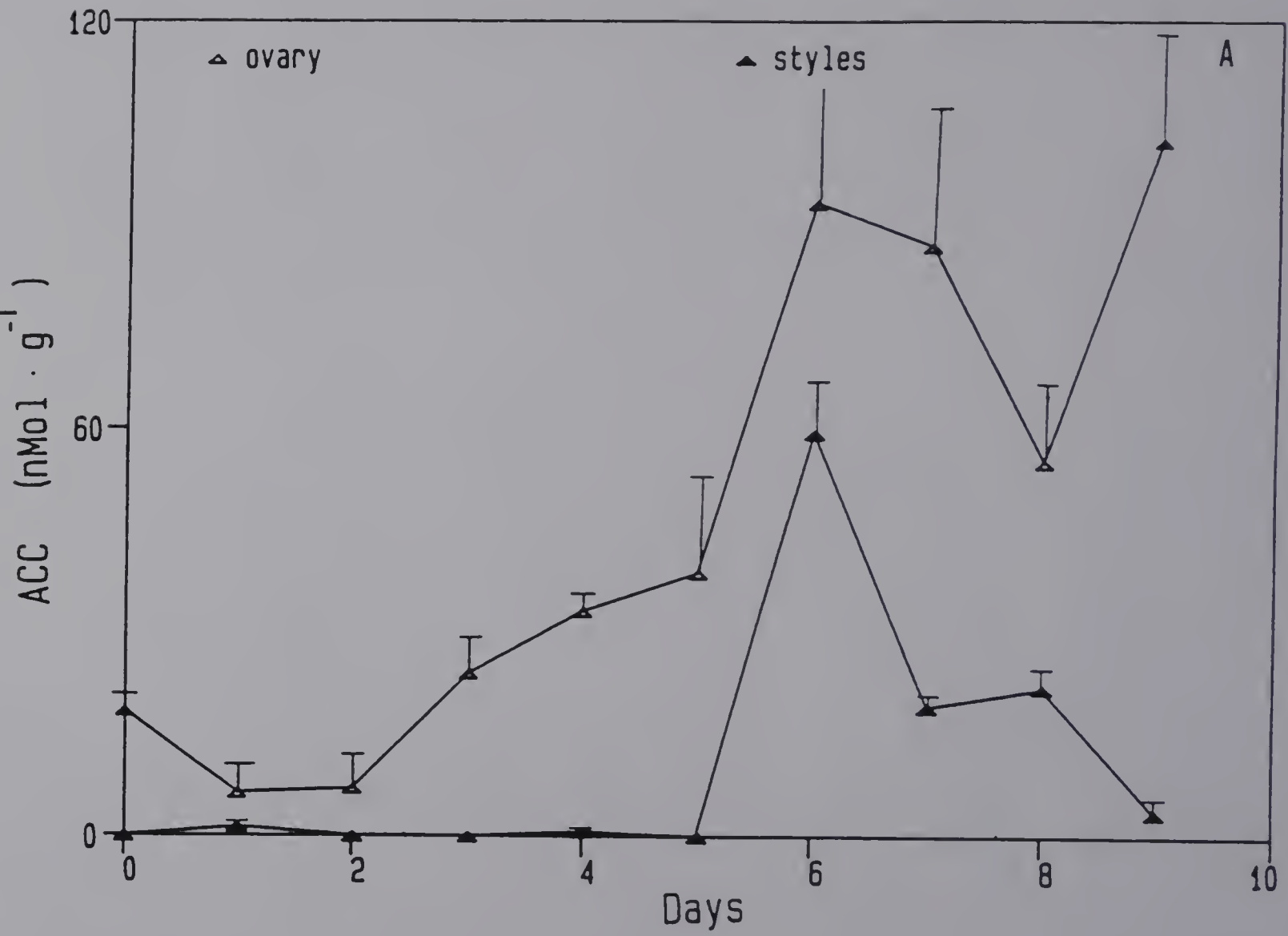
ACC and MACC Levels in Ageing Flower Parts.

All parts contained little to no ACC initially except for the ovary, which contained 5 to 15 nmol g^{-1} ACC (Figs. 4a and b). ACC concentration increased in all parts during ageing, and in the ovary and the styles it peaked at 6 d after opening (Fig. 4a). Both the receptacle and the petals produced a transient peak at 3 d after opening, which lasted 1 to 2 d, respectively (Fig. 4a). ACC in both peaked again at 7 d. The green tissue did not start producing significant amounts of ACC until after 5 d and its concentration then increased through 9 d (Fig. 4b). Levels of ACC remained high in all parts but the petals. Levels of MACC were synchronous in all parts (Fig. 5). They started increasing after 1 d, with a very large peak occurring at 3 d. At 4 d MACC levels had fallen to 70% of the peak except for the green tissue, which had decreased to almost zero. Another large peak of MACC was detected again at 7 d, decreasing at 8 d, with levels on the increase again by day 9.

Identity of Endogenous ACC and Conjugate.

Radiolabeled ACC came off the thin-layer plate between R_fs 0.20 and 0.30. Endogenous ACC also showed activity on the plate at this region. Endogenous MACC moved to the same spot on the plate as did authentic

Figures 4 a and b. Endogenous ACC concentrations in excised carnation flower parts from flowers harvested (cut) at 0 days and aged 0 to 9 days at 22 C under continuous light. The bars represent SE of four replications. a-ovary and styles. b-petals, receptacle, and green tissue.


120
60
0
ACC (nMol · g⁻¹)
ovary
styles
A
0
2
4
6
8
10
Days

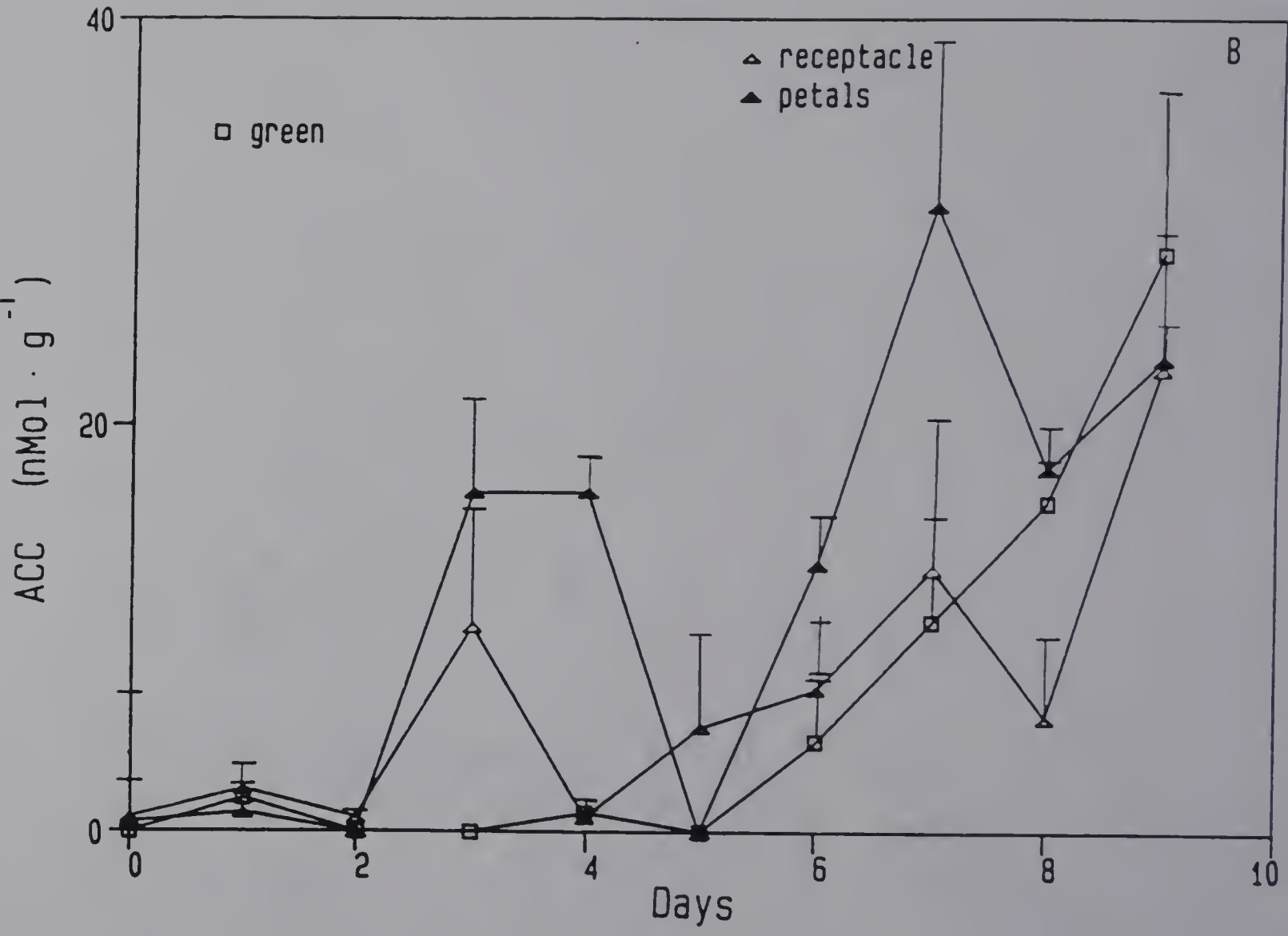
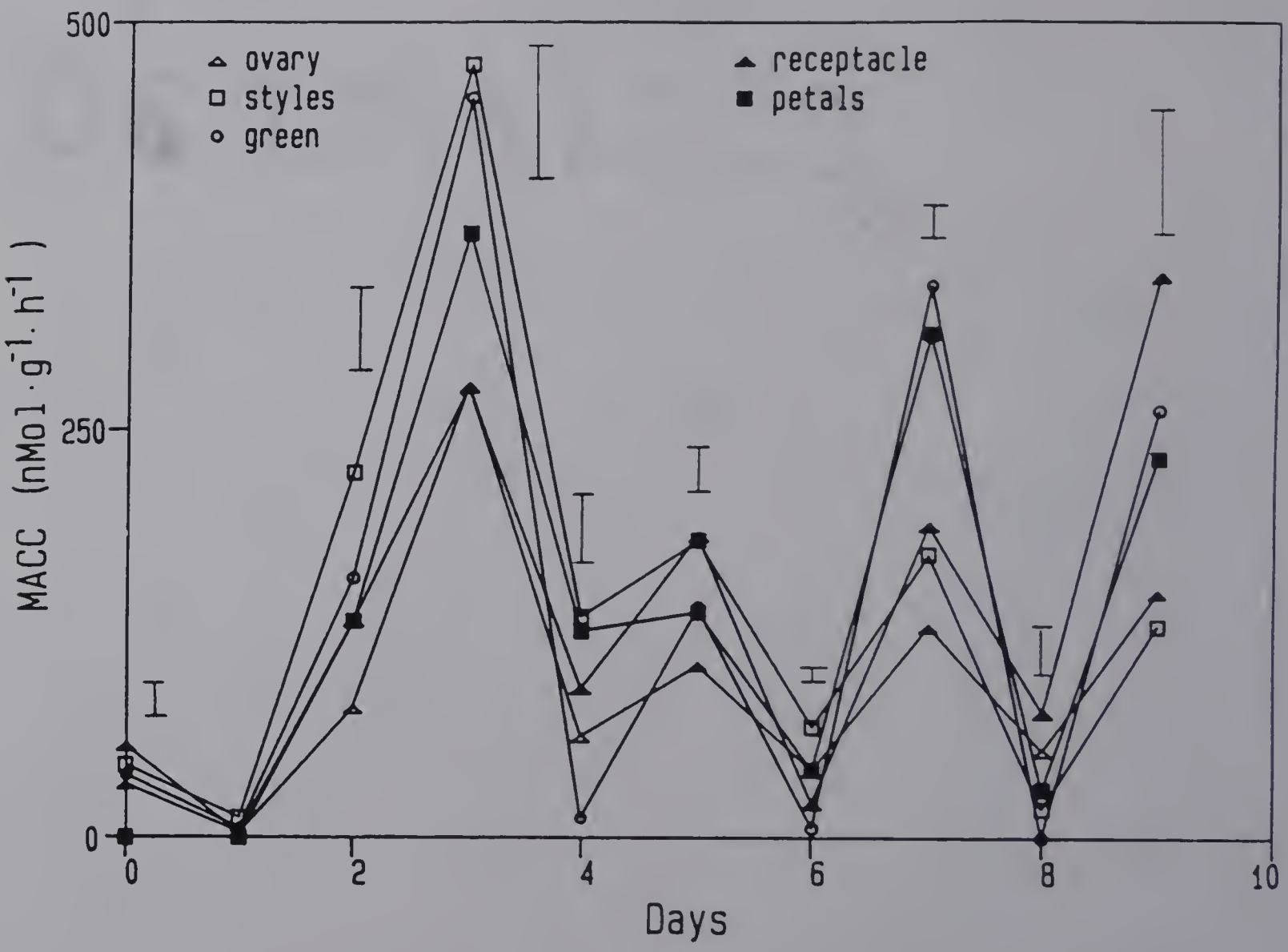

B
receptacle
petals
green
40
20
0
ACC (nMol · g⁻¹)
0
2
4
6
8
10
Days

Figure 5. Endogenous MACC concentration in excised carnation flower parts from flowers harvested (cut) at 0 days and aged 0 to 9 days at 22 C under continuous light. The bars either to the right or directly above data points indicate the pooled SE for each day of four replicates.


ovary
styles
green
receptacle
petals
500
250
0
MACC (nMol·g⁻¹·h⁻¹)
0
2
4
6
8
10
Days

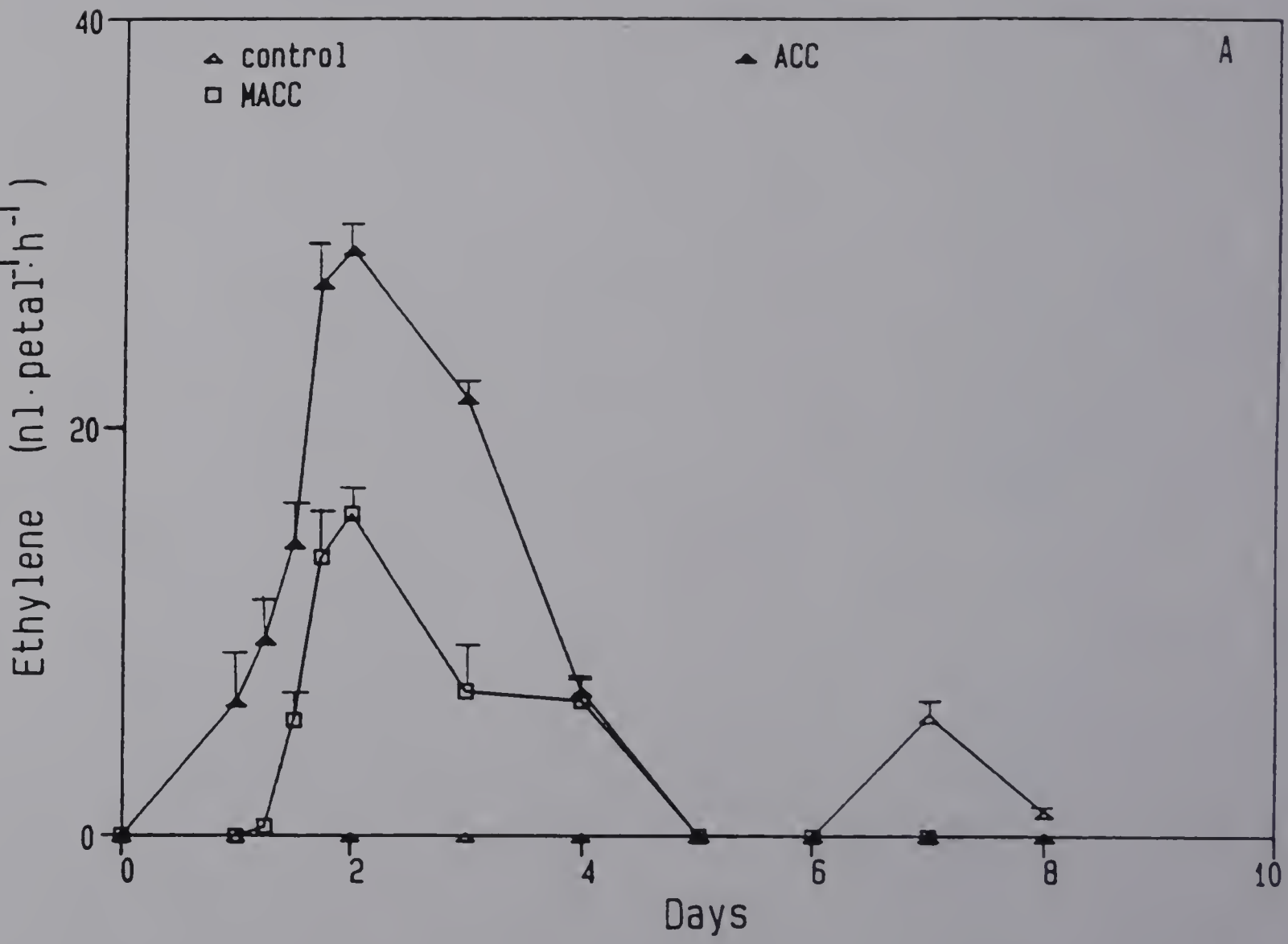
MACC, which was between Rfs 0.50 and 0.60. These results are similar to those obtained by others (Hoffman, et al., 1982, Lizada and Yang, 1979).

Excised Petal Experiments.

Exogenous applications of ACC and MACC both induced ethylene production in preclimacteric carnation flower petals (Fig. 6a). Ethylene production and subsequent petal inrolling occurred at about 28 h after incubation commenced in the ACC-treated petals. MACC-induced ethylene resulted in inrolling at about 36 h. About 87 % of applied material was taken up for both ACC and MACC (data not shown). Control petals did not start producing measurable ethylene until after 6 d, with inrolling commencing at 7 d. The response time for MACC-induced ethylene production was concentration-dependent (Table I). Petals treated with 0.25, 0.5, and 1.0 mM MACC inrolled at 4, 3, and 1.5 d, respectively.

Treating excised petals with ethylene inhibitors combined with 1.0 mM MACC resulted in a response similar to that observed by Jiao et al. (1986) in vegetative tissue. Petals treated with MACC + AVG produced ethylene and inrolled by 36 h (Fig. 6b), which is the same response as without AVG (Table I). Petals pretreated with STS, an inhibitor of ethylene action (Veen, 1979), and then fed MACC, produced measurable ethylene but did not inroll within 9 d (Fig. 6b). Those treated with MACC + 1.0 mM $CoCl_2$ in the feeding solution did not produce any measurable ethylene and still had not inrolled by 9 d (Fig. 6b). The cobalt response was concentration dependent; neither 0.1 nor 0.5 mM $CoCl_2$ inhibited MACC-induced ethylene production (Table I).

Figures 6 a and b. Production of ethylene in preclimacteric carnation petals. Arrows indicate first visible sign of petal wilting. Bars represent SE of three replicates. a- Petals treated with 1.0 mM ACC, MACC or distilled water. b- Petals treated with 1.0 mM MACC + 0.1 mM AVG, 1.0 mM $CoCl_2$ or STS.


40
20
0
control
MACC
ACC
A
Ethylene (nl·petal⁻¹·h⁻¹)
0
2
4
6
8
10
Days

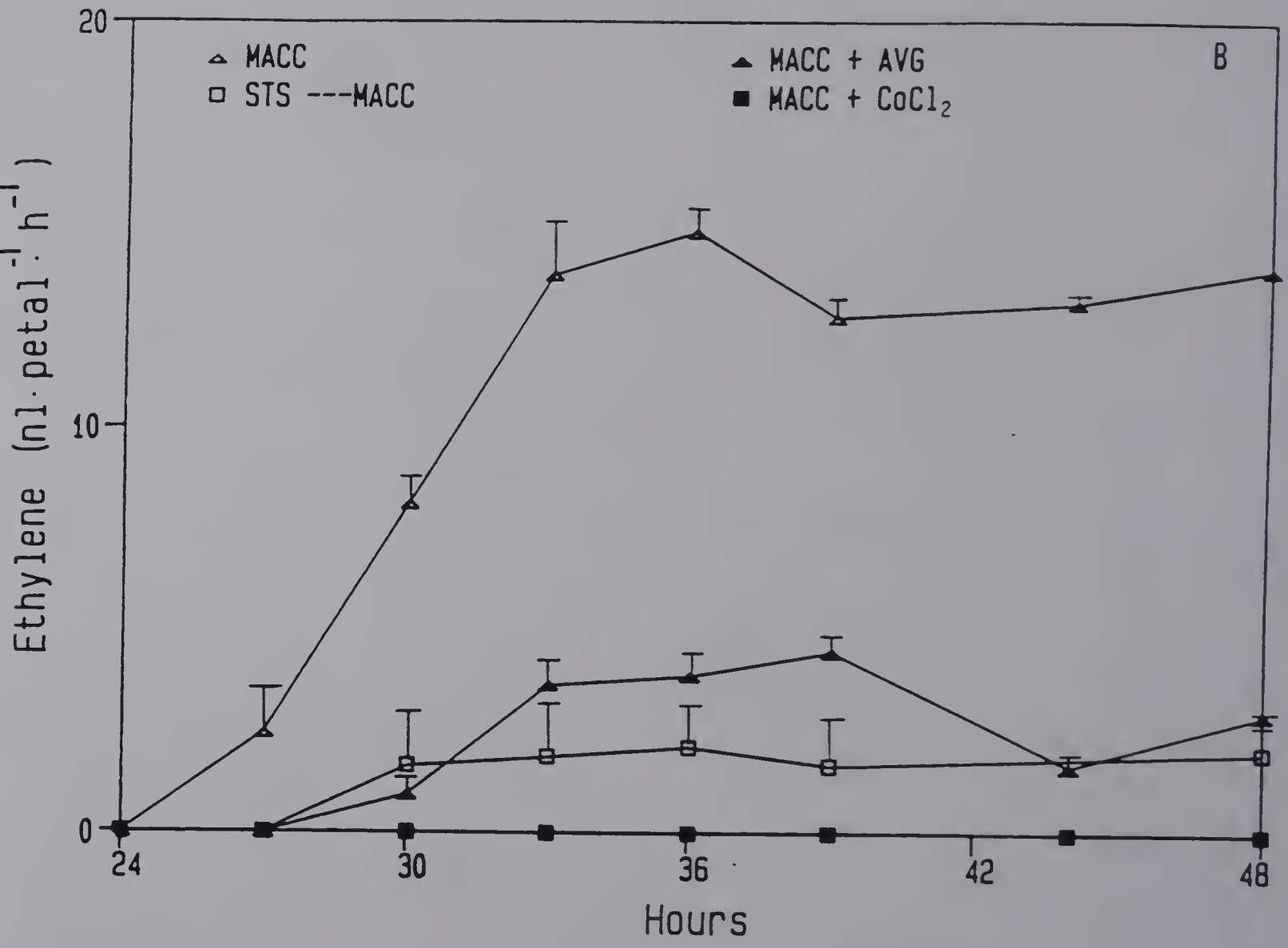

20
10
0
MACC
STS ---MACC
MACC + AVG
MACC + CoCl2
B
Ethylene (nl. petal-1 h-1)
24
30
36
42
48
Hours

Table I. Effects of different concentrations of MACC and $CoCl_2$ inhibitor on MACC-induced ethylene production in preclimacteric petals.

Treatment	Peak Ethylene Production	Days to Inrolling
	(nl petal^{-1} h^{-1})	
Control	15.6(2)	7.0
MACC (0.25 mM)	13.3(2)	4.0
MACC (0.50 mM)	19.8(0)	3.0
MACC (1.0 mM)	15.4(1)	1.3
MACC, 1 mM + 0.1 mM $CoCl_2$	14.4(3)	2.2
MACC, 1 mM + 0.5 mM $CoCl_2$	10.0(0)	2.0
MACC, 1 MM + 1.0 mM $CoCl_2$	0	---

Discussion

The synchrony of ethylene peaks observed in all flower parts in agreement with earlier work (Bufler, et al., 1980, Nichols, 1977). In addition, the same respiratory climacteric that was seen in the intact ageing flower (Bufler, et al., 1980) occurred for each individual flower part. It is interesting to note that although flower senescence is initially visualized as petal wilting, all the parts acted similarly in regard to ethylene and carbon dioxide production. Ethylene production rates in the different tissues coincided somewhat with the ACC concentrations. A rise in ACC occurred either just prior to the ethylene peak or coincided with it in all parts of the flower. In this experiment there was a transient peak in ACC concentrations in both the petals and the receptacle at 3 d. This did not result in an increase in ethylene production. Hsieh and Sacalis (1986) also reported a transient peak of ACC in the receptacle at this stage of development in the carnation flower. They suggested that this early peak may be a source of ACC for other parts of the flower. However, their data showed that concentrations of ACC in the petal bases and tops were higher than that found in the receptacle at 3 d, which is not consistent with their suggestion. The ACC peaks we observed did not result in any increased ACC or ethylene anywhere else in the flower after the peaks declined. We did detect a great deal of conjugated ACC in all parts at this stage, however. Perhaps the malonyltransferase enzyme responsible for conjugating ACC was saturated in the receptacle and the petals at this stage of development, which would have resulted in free ACC. Because so much MACC was found in all parts in the preclimacteric flower, it is

unlikely that mobilization of ACC from one part of the flower to another is necessary for the synchronous production of ethylene found throughout the flower.

Exogenous application of MACC induced abundent ethylene production in 1-d-old flower petals (Fig. 6a). This has been shown previously in vegetative tissue (Jiao, et al., 1986), but we are unaware of any previous report of it in reproductive tissue. MACC-induced ethylene production also resulted in accelerated senescence in the preclimacteric petal, as indicated by irreversible petal wilting. Through the use of specific ethylene inhibitors it was established that the MACC-induced ethylene in vegetative tissue was coming from the MACC and not from the synthesis of new ACC (Jiao, et al., 1986). We have now shown this to be true for petal tissue also. When the MACC feeding solution was amended with AVG, an inhibitor of ACC synthase, the petals still produced ethylene and senesced. When the MACC solution was amended with 1 mM $CoCl_2$, which inhibits ACC conversion to ethylene, petals did not produce any measurable ethylene by 9 d after opening. When we applied STS, an inhibitor of ethylene binding, as a dip prior to MACC treatment, the petals produced ethylene but did not wilt. Petal wilting is thought to be a consequence of ethylene binding (Veen, 1979).

Many different plant tissues have the ability to conjugate ACC to the malonylated form (Fuhrer and Fuhrer-Fries, 1985, Hoffman, et al., 1982, Knee, 1985, Satoh and Esashi, 1984 a and b). Generally, it has been regarded as an inactive endproduct of ACC synthesis (Hoffman, et al., 1983). In this experiment all parts of the flower contained peak levels of MACC 3 d after opening. This did not coincide with increased levels

of either ACC or ethylene. In the preclimacteric apple fruit, Mansour, et al.(1986) found levels of conjugated ACC about 10 times greater than levels of ACC. Satoh and Esashi (1984a) observed an accumulation of MACC in the developing cocklebur seed, also before any increase in ACC or ethylene. Perhaps the accumulation of conjugated ACC is a common phenomenon in developing reproductive tissue. The synchronous behavior of this metabolite during ageing may also have physiological significance. Bufler, et al. (1983) observed synchronous changes in polysome populations in ageing carnation flower parts. They noted this as an interesting observation due to the fact that the different flower parts are at very different stages of development during this time. The reduction of MACC in flower parts 4 d after opening was approximately 70 % in all parts except the green tissue, where the decrease was almost to zero. The synchronous behavior of all parts suggests a similar enzyme system working to hydrolyze the conjugate and metabolize the product to something other than ACC or ethylene. Presently, we are attempting to determine what that metabolite(s) might be. The peak of MACC at 7 d may represent conjugation of saturating levels of ACC. The synchronous reduction at 8 d, and then an increase again at 9 d, is difficult to explain at this juncture. Further research is needed to determine if some metabolite is being formed and reused in a cyclic nature, and if this holds any physiological significance for the different senescing flower parts.

C H A P T E R I I I

THE METABOLISM OF [2,3-^{14}C]ACC IN PRECLIMACTERIC CARNATION FLOWER PARTS

Introduction

The first visible sign of senescence in carnation (Dianthus caryophyllus L. cv. Scanea 3C) flowers is an irreversible wilting of the petals (Nichols, 1971). This wilting coincides with a surge of ethylene and a concomitant increase of 1-aminocyclopropane-1-carboxylic acid (ACC) in the tissue (Bufler, et al., 1980, Hanley, et al., 1988, Nichols, 1977). Little to no free ACC has been detected in either preclimacteric flowers (Bufler, et al., 1980, Hanley, et al., 1988, Woodson, et al., 1985) or fruits (Hoffmann and Yang, 1980, Mansour, et al., 1986). Considerable amounts of 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC), the primary conjugate form of ACC (Hoffmann, et al., 1982), have been found in developing reproductive tissues (Hanley, et al., 1988, Hoffman, et al., 1983, Peiser, 1986, Satoh and Esashi, 1984 a and b). In preclimacteric apples, the concentration of MACC was almost 10 times as much as that of the free ACC (Mansour, et al., 1986). Generally, endogenous MACC has been regarded as an irreversible, inactive endproduct of ACC synthesis (Hoffman, et al., 1983, Yang, 1985). However, in some plant tissues exogenous [^{14}C]MACC was hydrolyzed to ACC and ethylene production was induced (Jiao, et al., 1986). Exogenous application of MACC to excised, carnation flower petals resulted in the production of MACC-induced ethylene and senescence in the preclimacteric flower (Hanley, et al., 1988). Additionally, Matern, et al., (1984) reported, without presenting data, the isolation of an aminoacylase enzyme that increased as peanut plants senesced.

increased as peanut plants senesced.

When endogenous MACC levels were investigated in young, excised carnation flower petals, a large, transitory peak was detected four days prior to petal wilting and the ethylene climacteric (Hanley, et al., 1988). This hydrolysis of the ACC-conjugate did not result in any detectable ACC or ethylene, and it was concluded that endogenous MACC was being metabolized to some other product(s) in the preclimacteric flower.

Given the important role of ethylene and ethylene precursors in plant senescence, the objectives of this study were to (1) investigate the metabolism of radiolabelled ACC in preclimacteric carnation flower petals, and (2) identify some of its metabolites.

Materials and Methods

Plant Materials

Greenhouse-grown carnation flowers were harvested just prior to when petals formed a right angle to the stem, and placed individually in 50 ml test tubes containing 200 mg l^{1} Physan^{TM} (microbicide) solution. The flowering stems were kept under continuous fluorescent lighting at 22 C for 0 to 9 days. At each sampling day petals from the outermost whorl were excised. For petal segment experiments, 3-day-old petals were excised and only the proximal portion of the petal was used.

Chemicals

All extracting solvents were of analytical grade and purchased commercially. [2,3-^{14}C]ACC (80 uCi/uM) was purchased from Research Products International. Authentic MACC and anthocyanidin standards were gifts of Drs. S.F. Yang anf T. Boyle, respectively.

Radioactive Experiments

Eight to ten petal segments from 3 to 4 flowers were vacuum infiltrated (25 cm Hg) 2 to 3 times for 5 min each until all of the 5 ul (0.135 uCi) of [2,3-^{14}C]ACC (20 uCi/uM) was taken up into the tissue. After infiltration segments were rinsed with distilled water, blotted dry, and placed in 25 ml erlenmeyer flasks. Each flask was capped with a rubber septum and incubated in the light at 22 C for 4 or 6 h. A plastic well containing a paper wick wetted with 0.2 ml of 0.25 M $Hg(ClO_4)_2$ was hung in each flask to absorb evolved radiolabelled ethylene (Meir, et al., 1985). After incubation the petals were removed and frozen at -20 C. The mercuric perchlorate wicks were each placed in 20 ml scintillation vials with 10 ml scintillation solution (Scintiverse II, Fisher) and counted after soaking in the scintillant. Efficiency of the scintillation counter was approximately 84 % and background was between 60 and 80 CPM. Three- and four-day-old intact petals were placed individually in 20 ml vials and administered 10 ul (0.54 uCi) [2,3-^{14}C]ACC (40 uCi/uM). These were left uncapped in the light for 2 h or until all the radioactive solution had been taken up. Then, an additional 500 ul of distilled water was added to each vial. Each was capped with a rubber septum holding two plastic wells with wicks, one with $Hg(ClO_4)_2$ as described above and the other with 10 % KOH to absorb radiolabelled CO_2 (Meir, et al., 1985). The petals were incubated in the vials for 24 h in the light at 22 C. After incubation each petal was rinsed with distilled water, blotted dry, and frozen. Radioactivity in wicks for both ethylene and CO_2 were counted as described above. For both intact petals and petal segments the radioactivity taken up was

calculated by subtracting the activity in the water wash (including any remaining in the incubation vessel) from the original amount supplied. Both petal segments and intact petals were administered distilled water as a control and analyzed for production of ethylene after incubating up to 24 hours.

Extraction and Separation of [2,3-^{14}C]ACC Metabolites

Three- and four-day-old petals and 3-d-old petal segments were homogenized in 80 % ethanol using a hand-held homogenizer. The homogenates were centrifuged and decanted, and the pellet was re-extracted in 80% ethanol. After a second centrifugation the supernatants were combined, and reduced to a small aqueous vol under vacuum at 40 C. Radioactive metabolites of petal segments and intact petals incubated 6 h and 24 h, respectively, were separated on silica gel TLC plates and developed twice in butanol:acetic acid:water (BAW) (4:1:1.5, v/v) for 14 cm (Lizada and Yang, 1979). Plates were divided into 10 equal Rf fractions, scraped, and eluted with 50 % ethanol. Ethanolic fractions were reduced to aqueous and an aliquot of each was counted for radioactivity. In addition, chromatograms of 3-day-old intact petals incubated 24 h were assayed for radiolabelled ACC (Lizada and Yang, 1979) and MACC (Hoffman, et al., 1982) content and activity quantified as evolved [^{14}C]ethylene (Meir, et al., 1985). Aqueous aliquots from each Rf fraction were assayed and evolved ethylene was absorbed to $Hg(ClO_4)_2$-wetted wicks as stated above. Paper wicks were placed in scintillation vials with 10 ml scintillation solution and activity was counted. A known amount of [2,3-^{14}C]ACC was added to a replicate test tube to determine efficiency of assay.

Relative Mobility of Labeled ACC and MACC

To determine relative mobility of [2,3-^{14}C]ACC and authentic MACC on silica gel TLC plates, each was either co-chromatographed or developed parallel to the crude petal extracts. Relative mobility of labeled ACC was determined after dividing the chromatogram into ten equal Rf fractions and analyzing each fraction for radioactivity. The location of unlabeled MACC on silica gel TLC plates was determined after analyzing each fraction according to the procedure of Hoffman, et al. (1982).

Separation by Ion Exchange and TLC

Three-day-old intact petals and petal segments were administered [2,3-^{14}C]ACC and homogenized as outlined above. The radioactive metabolites were first separated by passing the crude petal extract through a column (1.5 x 8 cm) of cation exchange resin Dowex 50 (H+ form) and washed with 2 bed vols of distilled water. The effluent was reduced to a small vol under vacuum at 50 C and a 250 ul aliquot was placed in a 20 ml scintillation vial with 10 ml scintillation solution, and radioactivity was counted. The remaining acidic effluent was saved for further separation. The basic fraction of the petal extract was eluted with 4 column vols of 2 N NH_4OH, reduced to a small vol under vacuum at 50 C, and radioactivity counted. No difference in efficiency of the scintillator due to quenching was detected in samples imparting a small amount of color, and efficiency was still approximately 84 %. The acidic effluent was then passed through an anion exchange column (1.5 x 5 cm) of Dowex 1-8 (OH- form), washed with 2 bed vols of distilled water, and eluted with 5 bed vols of 6 N formic acid (Hoffman, et al., 1983a). The formic acid eluate from the column was brought to a small vol under

vacuum at 50 C and a 250 ul aliquit was taken to measure radioactivity. A portion of both the acidic and the basic column eluates and of the acidic effluent were chromatographed on silica gel TLC plates as outlined above.

Pigment Experiments

Three-day-old intact petals were administered [^{14}C]ACC and incubated 24 h. After extraction in 80 % ethanol, the pigment fraction was separated on silica gel TLC plates as outlined above. The pigment fraction was detected on the plate as a reddish line at about Rf 0.35. The whole Rf band (0.3 to 0.4) was eluted from the plate with 50 % ethanol, reduced to aqueous under vacuum, and an aliquot was analyzed for radioactivity. The eluted aqueous pigment band (0.3-0.4) was then made to 2 N with HCL and heated in a covered test tube for 40 min at 100 C (Harborne, 1967). The acidic hydrolysate was washed twice with equal vols of ethyl acetate. An aliquot of the organic fraction was analyzed for radioactivity, and the washed aqueous fraction was partitioned against a small vol of isopentyl alcohol (Harborne, 1984). After partitioning, the pigmented alcohol fraction was pipetted off and aliquots of both the colorless aqueous and the colored alcohol fractions were analyzed for radioactivity. The remaining pigmented alcohol fraction was streaked across silica gel TLC plates, developed in BAW (4:1:1.5, v/v) (Harborne, 1984) and co-chromatographed with the anthocyanidin standards pelargonidin and cyanidin.

Excised carnation flower petals harvested 0 to 9 days after opening were homogenized twice in 2 ml ethanol (1% HCL) (Harborne, 1967) as outlined above. Aliquots of the ethanolic extracts were analyzzed for

pigment production using a spectrophotometer at 520 nm (Trippi and Paulin, 1984).

Results

Relative Mobilities of ACC and MACC

When radiolabelled ACC standard was developed on silica gel TLC plates, the activity was eluted from Rf 0.2. When authentic unlabeled MACC was developed on silica gel plates and activity quantified according the method of Hoffmann, et al. (1982), a portion of the MACC was detected in both Rfs 0.5 and 0.6. Thus, both Rf 0.5 and 0.6 bands were eluted from the plates and used to quantify MACC activity in the petal extracts.

Chromatographic Separation of [2,3-^{14}C]ACC Labeled Crude Extracts

Crude extracts of petal segments and intact petals administered [^{14}C]ACC and incubated 6 or 24 h were developed on silica gel TLC plates. After incubating for 6 h, 86 % of the activity co-chromatographed with standard [^{14}C]ACC (Table II). After 3- and 4-day-old petals were incubated 24 h, ACC accounted for only 36 and 14 percent, respectively, of the applied label. A small proportion of the radioactivity was recovered as conjugated ACC between Rfs 0.5 and 0.6, which did not vary greatly with increasing incubation time or petal age (Table II). In addition to co-chromatographing with labeled ACC and authentic MACC standards, all 10 Rf fractions on the chromatogram were assayed for both metabolites, which were quantified as evolved [^{14}C]ethylene (Table III). Radiolabeled ethylene was recovered from Rf 0.2 as ACC, and activity was detected in both Rf 0.5 and 0.6 after assaying for MACC (Table III). No ACC or MACC was detected in any of the other Rf fractions on the

Table II. Distribution of label following treatment of carnation petals with [2,3-^{14}C]ACC.

	Rf					
Conditions	0.1	0.2	0.3	0.4	0.5-0.6	0.7-1.0
	(% Radioactivity)					
3-d segments, 6h	4(1)	86(1)	3(0)	2(0)	4(0)	1(0)
3-d petals, 24h	6(1)	35(6)	8(3)	39(6)	10(3)	2(1)
4-d petals, 24h	7(0)	14(1)	10(1)	63(2)	5(2)	1(0)

Table III. ACC and MACC activity in metabolites of crude petal extracts.

Rf	Evolved [^{14}C]ethylene (CPM)	[^{14}C]ethylene/Rf (%)
0.1	0	0
0.2	10,004	82 (ACC)
0.3	0	0
0.4	0	0
0.5	3,001	96 (MACC)
0.6	1,886	72 (MACC)
0.7-1.0	0	0

chromatogram. The pigment fraction was visible on the chromatogram as a reddish band in the middle of Rf 0.4. The percent radioactivity associated with the pigmented Rf band increased markedly with increasing incubation time and petal age (Table II). After incubating 24 h, Rf 0.4 of 3-and 4-day-old petals contained 39 % and 63 % of the radioactive metabolites, respectively. A similar response was detected in 3- and 4-d-old petals after administering labeled MACC (Appendix, Fig. 19). Petal segments or intact petals that were administered a water control did not produce any detectable ethylene after incubating 6 or 24 h (data not shown).

Ion Exchange and Thin-Layer Chromatographic Separation of [2,3-^{14}C]ACC Metabolites

After 3-day-old petals were administered labeled ACC and incubated in the light for 4 to 24 h, a small percent of radioactivity that had been taken up was detected as either labeled ethylene or CO_2 (Table IV). The percent of labeled ethylene increased with increasing incubation time. Trace levels of the radioactive CO_2 were detected after incubating 24 h. When aliquots of the crude petal extracts were separated by cation exchange columns, more than 90 % of the applied activity remained in the basic (ACC-containing) eluate after incubating 4 or 6 h (Table IV). After 24 h this was reduced to approximately 68%, with a corresponding increase detected in the acidic effluent (MACC-containing). After passing the acidic effluent through an anion exchange column, most of the activity (95%) was retrieved in the formic acid eluate. Approximately 20 percent of the activity between Rf 0.1 to 0.4 in the acidic effluent was lost during anion column separation. This was determined to be ACC

Table IV. The metabolism of [2,3-^{14}C]ACC in carnation petals.

Incubation (h)	Ethylene	CO_2	Basic Eluate	Acidic Effluent
		% Radioactivity		
4	0.1(0)	---	96.5(1)	3.5(1)
6	0.2(0.1)	---	92.9(2)	7.1(2)
24	1.2(0.1)	0.3(0)	68.5(4)	31.5(4)

contamination in the acidic effluent (data not shown). After further separating the radiolabelled metabolites on silica gel TLC plates, approximately 60 % of the radioactivity in the formic acid residue co-eluted with authentic MACC in Rfs 0.5 and 0.6 (Table V). Activity was also detected between Rfs 0.7 and 0.8 but was not characterized. About 20 % of the radioactivity in the basic eluate co-eluted with authentic ACC on the chromatogram at Rf 0.2 (Table V). The pigment fraction was visible as a reddish line in Rf 0.4 and comprised about 6 % of the radioactivity of the basic fraction. Significant activity was detected at both Rfs 0.3 and 0.5, and was not characterized.

Radioactivity Associated with the Unglycosylated Pigment Fraction

To further determine whether or not the radioactivity detected in the pigment fraction (Rf 0.4) of the crude petal extract was associated with the anthocyanins, 3-d-old petals were administered labeled ACC and the extracted as outlined above. After chromatographing the crude extract, the colored pigment fraction was detected in Rf 0.4 and eluted. The eluate was acid hydrolyzed to yield the aglycone form of the pigments. After the acidic hydrolysate was washed with ethyl acetate to remove any flavone structures (Harborne, 1984), approximately 1% of the radioactivity was reclaimed in the organic wash fraction (Table VI). After washing, the aqueous hydrolysate was then partitioned against a small vol of isopentyl alcohol to separate the pigments. Six percent of the radioactivity was recovered in the anthocyanidin fraction (Table VI). The majority of the radioactivity detected in Rf band 0.4 remained in the colorless aqueous fraction. After the anthocyanin fraction was separated on a silica gel TLC plate, little to no activity was detected

Table V. TLC of petal extracts after fractionation by ion exchange columns.

Rf	Percent Radioactivity
Ammonia Eluate	
0.1	3(1)
0.2	19(2)
0.3	54(5)
0.4	6(1)
0.5	17(6)
Basic Effluent	
0.1-0.4	29(5)
0.5-0.6	41(3)
0.7-0.8	25(7)
0.9-1.0	4(2)
Formic Acid Eluate	
0.1-0.4	5(2)
0.5-0.6	59(5)
0.7-0.8	28(6)
0.9-1.0	8(1)

Table VI. Total radioactivity from the TLC pigment fraction.

Extract	CPM	Percent Radioactivity
Acidic Hydrolysate	98,000	100
Ethyl Acetate Wash	966	1
Isopentyl Alcohol	5,894	6
Aqueous Discard	91,140	93

from the lower half of the chromatogram (Figure 7). A dark, brownish streak was visible at Rf 0.9. This co-chromatographed with the pigment standard pelargonidin. Radioactivity was also detected at Rf 0.7, which were also observed in Rfs 0.6 and 0.8. The comman anthocyanidins, columnidin (orange-red) and peonidin (magenta), are recovered in Rfs 0.6 and 0.8, respectively, using BAW as the solvent (Harborne, 1967).

Determination of Endogenous Pigment Production

Ageing carnation petals did not start accumulating anthocyanins until day 2 (Figure 8). A steady increase was detected from 2 to 4 days. A large peak of anthocyanin production was observed at 7 days, with a subsequent decrease at 8 days coincident with petal wilting.

Discussion

The cyclic amino acid ACC is the immediate precursor to ethylene biosynthesis in higher plant tissue (Adams and Yang, 1979). N-malonyl ACC has also been identified as a major conjugate form of ACC (Hoffman, et al., 1982). Many plant tissues produce large quantities of ethylene after administering exogenous ACC, but some preclimacteric tissues produce only a small amount of ethylene after treatment with ACC (Hoffman and Yang, 1980, Yang, 1985). Very little is known about the metabolism of ACC when it is not either oxidized to ethylene or converted to the conjugate. In this investigation we examined the metabolism of exogenous [2,3-C^{14}]ACC applied to young carnation flower petals, which contain little to no endogenous ACC and produce little ethylene (Bufler, et al., 1980, Hanley, et al., 1988, Peiser, 1986). As outlined in Figure 9, within 24 h after application, ACC was metabolized to products other than

Figure 7. Chromatographic separation of isolated radiolabelled petal pigment fraction. Relative mobility of the anthocyanidin standards cyanidin (CY) and pelargonidin (PE) are indicated.

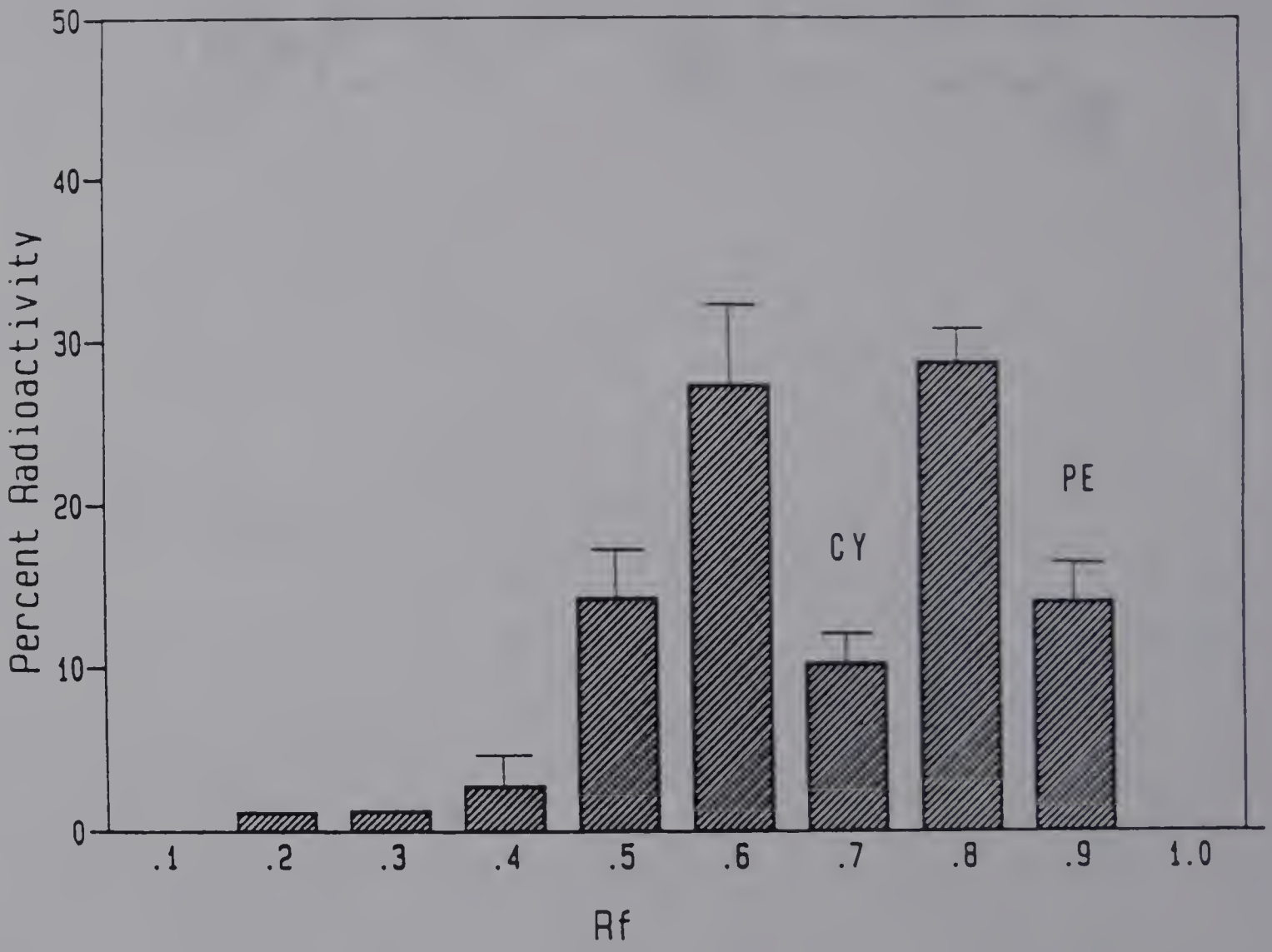

Percent Radioactivity
50
40
30
20
10
0
CY
PE
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
Rf

Figure 8. Endogenous concentration of anthocyanins in ageing carnation flower petals. Standard error bars are indicated (n=3).

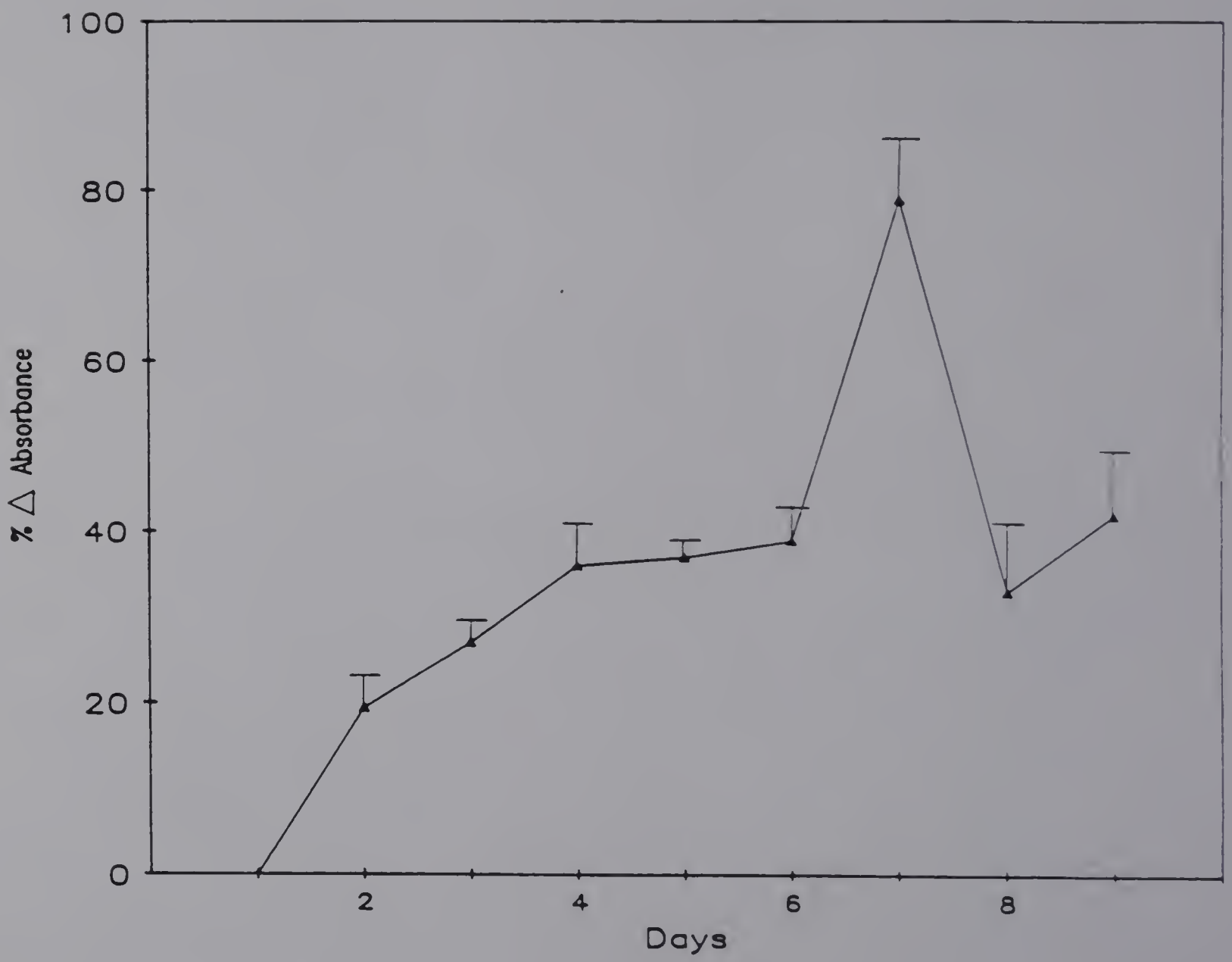
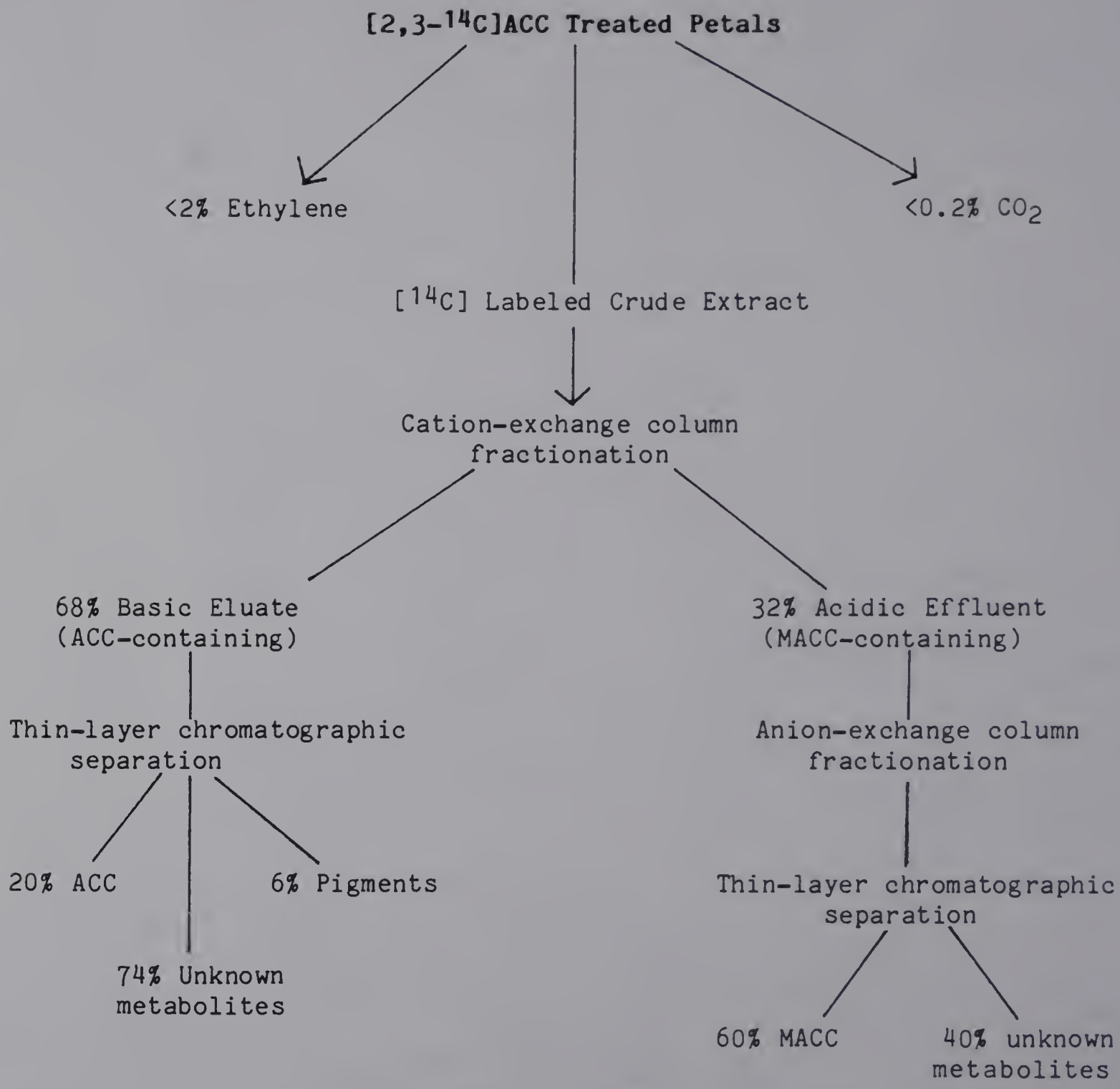

100
80
60
40
20
0
% Δ Absorbance
2
4
6
8
Days

Figure 9. Distribution of percent radiolabelled metabolites in carnation petals after incubating 24 h.


[2,3-14C]ACC Treated Petals
<2% Ethylene
<0.2% CO2
[14C] Labeled Crude Extract
Cation-exchange column fractionation
68% Basic Eluate (ACC-containing)
32% Acidic Effluent (MACC-containing)
Thin-layer chromatographic separation
20% ACC
6% Pigments
74% Unknown metabolites
Anion-exchange column fractionation
Thin-layer chromatographic separation
60% MACC
40% unknown metabolites

ethylene or the conjugate form in the preclimacteric petals. After the petals were administered radiolabeled ACC and incubated, less than 2% of the radioactivity taken up by the tissue was detected as labeled ethylene (Table III). These results concur with earlier findings that there is little activity of the ethylene-forming enzyme in preclimacteric carnation flowers (Manning, 1981, Mor and Reid, 1981, Whitehead, et al., 1984a). It has been proposed that the low activity of this enzyme which oxidizes ACC to ethylene may, in part, be due to strong competition with the malonyltransferase enzyme for the ACC substrate (Kionka and Amrhein, 1984). Large concentrations of endogenous MACC have been detected in preclimacteric apples (Mansour, et al., 1986), germinating seeds (Hoffman, et al.,1983, Satoh and Esashi, 1984a), and carnation flower parts (Hanley, et al., Peiser, 1986). However, in the present study, only a small percent of the radioactivity was recovered as the ACC conjugate (Table II). This is in contrast to germinating peanut seeds, which converted more than 90 % of the applied [2-^{14}C]ACC to [^{14}C]MACC after imbibition for 30 h (Hoffman, et al., 1983a). In a previous study we found that 3-day-old carnation petals contained approximately 45 nM endogenous MACC (Hanley, et al.,1988). In this investigation each petal was administered approximately 13 nM exogenous [^{14}C]ACC. Thus, the isotope would have been diluted to about 1/3 of the specific activity, which would have resulted in less than 20 % of the applied [^{14}C]ACC going to the conjugate form. These results suggest that the low amount of radioactive ethylene produced by these petals (Table I) does not appear to be the result of a high rate of malonylation of the exogenous [2,3-^{14}C]ACC applied.

Trace levels of labeled CO_2 that were detected in petals after incubating 24 h may be due to contamination of the isotope on the carbon 4 of the carboxyl group. Our isotope was labeled on C-2 and C-3. Peiser, et al. (1984) found that oxidation of ACC to ethylene yielded CO_2 derived from the carboxyl group. A similar oxidation mechanism of aminoisobutyric acid (AIB), an ACC analog, was proposed yielding CO_2 from the C-1 carboxyl group (Liu, et al., 1984).

In this study the metabolism of exogenous ACC in carnation flower petals was time-dependent. After incubating either 4 or 6 h most of the radiolabel was still in the form of nonmetabolized ACC (Table II). Liu, et al. (1984) obtained similar results when they administered [^{14}C]AIB to mungbean hypocotyls; after 6 h most of the label was retrieved as nonmetabolized AIB and a small amount as MAIB conjugate. After flower petals were allowed to incubate 24 h, the majority of the [^{14}C]ACC taken up by the petal tissue had been metabolized (Table II).

The metabolism of exogenous [U-^{14}C]ACC has been studied in whole apple fruit (Knee, 1985). Fruits were administered labeled ACC, incubated 24 h, and radiolabeled metabolites analyzed. Regardless of time of harvest, between 30 and 40 % of the radioactive ACC was recovered as an acidic metabolite, which was experimentally confirmed to be MACC. After 24 h approximately 60 % of the basic fraction was recovered as nonmetabolized ACC. In addition to recovery of both ACC and MACC, radioactivity was also retrieved from a neutral fraction and a minor basic metabolite, but these fractions were not characterized.

The large peak of endogenous pigment production at 7 days (Figure 8) is coincident with the ethylene climacteric (data not shown). Ethylene

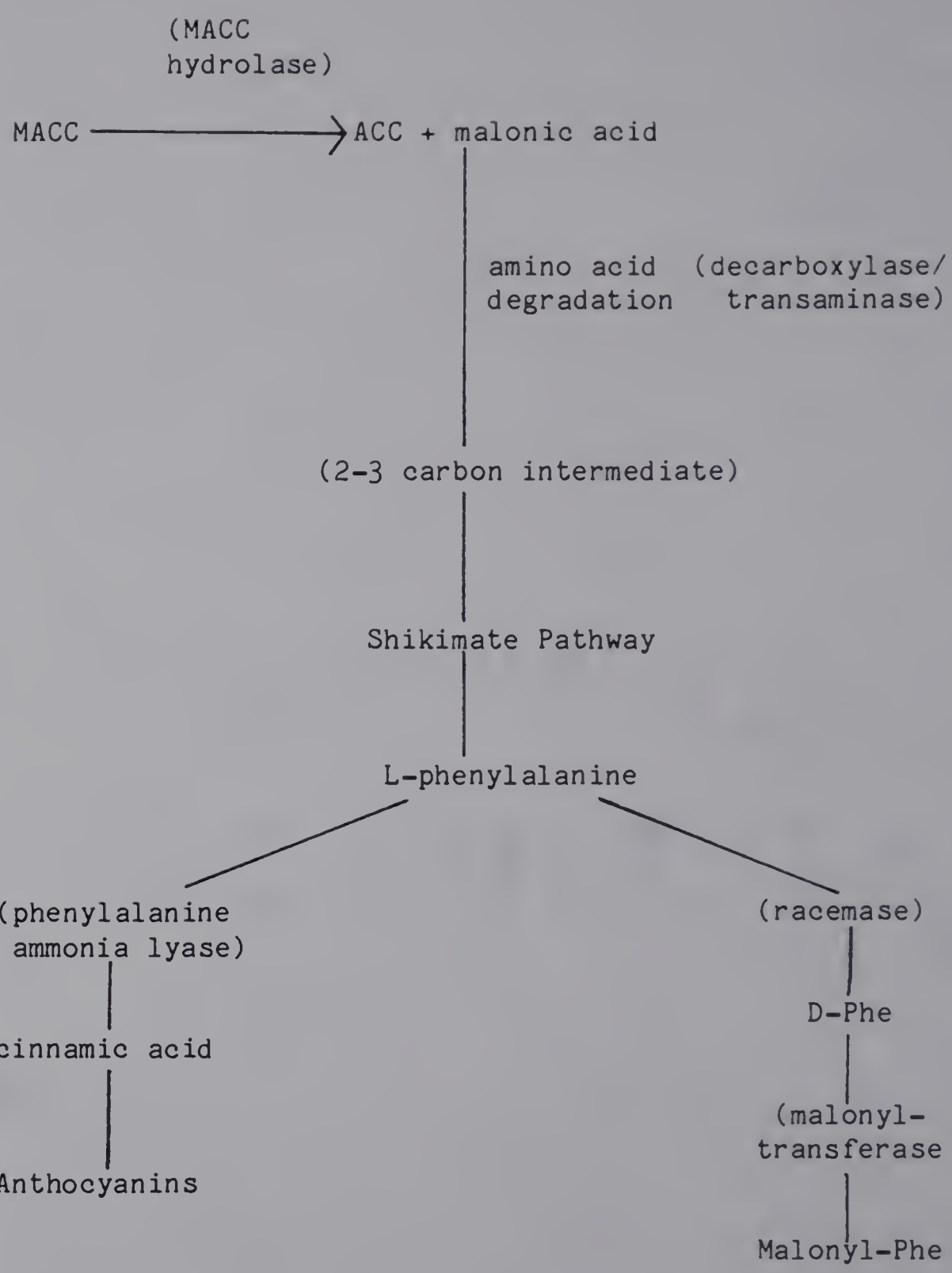
has been implicated in the promotion of the anthocyanin biosynthesis enzyme phenylalanine ammonia-lyase (PAL) (Faragher, 1983). A synchronous peak of activity was detected between the PAL enzyme and ethylene in ripening apple fruit skin (Blankenship and Unrath, 1988). The linear increase of endogenous anthocyanins in the preclimacteric petal (Figure 8), was not associated with any increase in ethylene production (data not shown). The shunting of labeled ACC metabolites into pigment formation (Table IV) was also not associated with increased ethylene biosynthesis, indicating that anthocyanin synthesis was related to some mechanism other than induction of the PAL enzyme by ethylene. Chappell, et al., (1984), observed pathogen-induced ACC-synthase (ethylene), chitinase, and PAL enzyme in cultured parsley cells. The peak of ACC-synthase activity preceded an increase of induced PAL enzyme by several hours. They were not able to induce the PAL enzyme when cells were treated with either exogenous ACC or ethylene. They concluded that the induction of the PAL enzyme may not be related primarily to the production of ethylene by the cells, but rather by some mechanism controlling the activity of the enzymes involved in ethylene biosynthesis. The increase of endogenous pigments occurring at 8 days concurs with earlier work which found rapid pigment exudation coincident with petal wilting (Trippi and Paulin 1984).

To our knowledge this is the first demonstration of metabolites of ACC going to anthocyanin production. Anthocyanins are synthesized via condensation of three molecules malonyl CoA and one p-coumaryl group derived from acetyl CoA and shikimic acid, respectively, (Goodwin and Mercer, 1983). Shikimic acid is also the precursor of many aromatic amino acids, including phenylalanine (Phe) (Zubay, 1983). D-amino acids

specifically, D-Phe, are competitive inhibitors of the malonylation of ACC, resulting in free ACC and conjugated D-Phe (Liu, et al., 1983, Liu, et al., 1984). The same malonyltransferase enzyme can conjugate both ACC and D-Phe, with Km values of 0.15 mm and 0.8 mM, respectively (Su, et al., 1985). The catabolism of ACC may be resulting in metabolites entering the shikimic acid pathway to produce phenylalanine (Fig. 10). At this juncture the amino acids could either be utilized in flavonoid production as catalyzed by the phenylalanine ammonia-lyase (PAL) enzyme, or conjugated by the malonyltransferase enzyme to form M-Phe. Because onlya small percent of the radioactivity was recovered in the anthocyanin fraction (Tables V and VI), the primary function of ACC metabolism is not the production of pigments in the preclimacteric carnation flower petal. If the principal intermediate of ACC catabolism is Phe, the amino acid may be a potential substrate for the malonyltransferase enzyme, which might otherwise be using ACC as a substrate.

Since increased ethylene concentrations frequently occur prior to the onset of senescence in many plant tissues, it is important to determine the conditions influencing its production. The relative activity of the enzymes synthesizing and conjugating ACC, and the activity of the ethylene-forming enzyme, have been regarded as the main factors involved in the control of ethylene synthesis (Yang, 1981). This investigation has demonstrated that ACC is metabolized to products other than those involved in ethylene biosynthesis. When we review the relative importance of the 1-aminocyclopropane-1-carboxylic acid metabolite in senescing and presenescing tissue, its catabolism to products other than

Figure 10. MACC metabolism in the preclimacteric carnation flower petals.


(MACC
hydrolase)
MACC
ACC + malonic acid
amino acid degradation
(decarboxylase/
transaminase)
(2-3 carbon intermediate)
Shikimate Pathway
L-phenylalanine
(phenylalanine
ammonia lyase)
cinnamic acid
Anthocyanins
(racemase)
D-Phe
(malonyl-
transferase
Malonyl-Phe

ethylene may serve as a potential modulator of ethylene production and senescence. This may be particularly important in tissues containing a large amount of MACC early in development.

C H A P T E R I V

ENDOGENOUS LEVELS OF ABSCISIC ACID IN AGEING CARNATION FLOWER PARTS

Introduction

Accelerated ethylene evolution is involved in the senescence of carnation flower petals; its deleterious effects are related to an alteration of cell membrane composition and integrity (Thompson, et al., 1982). However, the event(s) that trigger the onset of increased ethylene production are still unknown. Abscisic acid (ABA), as well as ethylene, is involved in promoting the physiological events of senescence (Mayak and Halevy, 1981), but the relationship between these two hormones in promoting flower senescence is not well understood. When exogenous ABA was administered to cut carnation flowers, the ethylene climacteric rise and subsequent petal wilting were both advanced by two days (Ronen and Mayak, 1981, Mayak and Dilley, 1976). This response was prevented by treatment with aminoethoxyvinylglycine (AVG), an inhibitor of ethylene biosynthesis (Ronen and Mayak, 1981) or by hypobaric ventilation of the ethylene produced (Mayak and Dilley, 1976). Thus, it was concluded that exogenous ABA was stimulating the production of ethylene. In detached carnation petals an increase of endogenous ABA paralleled the ethylene climacteric rise and consequent petal wilting (Eze, et al.,1986). Nowak and Veen (1982) detected a large increase of ABA in the carnation pistil and a small increase in the petals two days prior to the ethylene surge. When they pretreated these same flowers with silver thiosulfate complex (STS), an inhibitor of ethylene action (Veen, 1979), the increase of ABA was completely prevented. Other studies have reported an inhibition of

both cytokinin accumulation (Van Staden and Dimalla, 1980) and gynoecium enlargement (Dimalla and Van Staden, 1980) in senescing carnations pretreated with STS.

Immunological analysis is now a recommended method in the quantification of ABA and related compounds (Hirai, 1986). The enzyme-linked immunosorbent assay (ELISA) is based on the competition between free ABA in the sample and an alkaline-phosphatase labeled ABA tracer for a fixed number of high-affinity antibody binding sites (Weiler, 1984).

In this study we attempted to determine whether or not any correlation exists between the concentration of ABA in the plant tissue and the ontogeny of the individual flower parts. Through the use of a sensitive monoclonal antibody (McAb), quantitative ABA measurements could be made on small tissue samples. In this way, we hoped to elucidate a possible predisposition of tissues for ethylene synthesis.

Materials and Methods

Plant Material

Greenhouse-grown carnation flowers (Dianthus caryophyllus L. cv Scanea 3C) were harvested when outer petals first started to form a right angle to the stem, as described previously (Hanley, et al., 1988), and were kept in continuous light at 22 C for 0 to 9 days. At each day, petals (five from the outer whorl) and green tissue (including the calyx, leaves and 12 cm of stem) were excised, and fresh weights and ethylene production (petals) were recorded. Subsequently, the petals and green tissue were frozen at -20 C, lyophilized, and saved for ABA analysis. To evaluate the endogenous ABA levels in the remaining flower parts, at each day the ovary, receptacle, and styles (including the stigmatic surface)

were excised, fresh weights recorded, and flower parts were frozen at -20 C.

Chemicals

All ABA extraction and purification solvents were of HPLC grade and purchased commercially. RS-(-/+)ABA was purchased from Sigma. Disposable silica gel columns were from Baker. [2-^{14}C]ABA (8 uCi/uM) was from Amersham. Phytodetek^{TM}-ABA monoclonol antibody kits were from Idetek, San Bruno, CA. The antibody was raised against S-(+)ABA attached to the carrier protein, bovine serum albumin (BSA) at the ketone group, resulting in an antibody that is highly specific for the free, physiologically active ABA (Quarrie, et al., 1988).

Determination of Ethylene

At each sampling day excised petals were placed in 25 ml flasks that were sealed with rubber septa. After incubation in the light for one hour, ethylene was determined from a 1 ml gas sample withdrawn from the headspace using a 3 ml disposable syringe. Gas samples were injected into a Shimadzu GC-8A gas chromatograph equipped with an activated alumina column and flame ionization detector. Data were compiled using a Shimadzu GC integrator. Immediately after ethylene measurements, petals were frozen, lyophilized, and ground using a Wiley mill (20 mesh screen).

ABA Extraction and Purification Procedure

Generally, a preliminary purification of the tissue sample is advised as a precaution against substances in the crude extract interfering with the activity of the antibody (Pengelly, 1977). However, in other studies the use of this antibody precluded the need for prior purification of the crude extract, and very similar levels of sensitivity were observed

between the immunoassay technique and physico-chemical methods of detection (Mertens, et al., 1983, Walker-Simmons, 1987). In this study a modified version of Subbiah and Powell (1987) for ABA extraction and purification was utilized. At each sampling day frozen styles, ovaries, and receptacles were homogenized twice in 5 ml methanol:acetic acid:water (80:19:1) (including 20 mg/ L BHT as an anti-oxidant (Maldiney, et al., 1986) using a hand-held homogenizer. Lyophilized, 50 mg samples of petal and green tissue were extracted twice in 10 ml of extracting solvent at 4 C for 24 h. Homogenates and tissue extracts were centrifuged at 10,000 g; supernatants were combined and placed in 25 ml beakers. Crude extracts of petals, styles, and green tissue were each administered 25 ul (0.02 uCi/ul) [2-^{14}C]ABA (S.A.= 8 uCi/uM) for estimation of recovery at each purification step. Methanol was evaporated from the supernatants in the dark under the strong draft of a hood. Aqueous extracts were placed in 20 ml plastic scintillation vials; pH was adjusted to 3.0 with 0.5 M acetic acid and the extracts were partitioned three times against diethyl ether. Preliminary examination revealed the recovery of varying quantities of radiolabeled ABA tracer in the emulsion formed between the aqueous and organic fractions after partitioning. To dissipate this emulsion, after each partitioning steps the vials were capped and centrifuged at 5000 g for 10 min. The organic fractions were collected and made to a final volume of 1:1, ether:hexane (v/v) (ET:HX). Disposable, pre-packed, Baker-SPE 3 ml silica gel columns were preconditioned by first aspirating through 2 column volumes of methanol, followed by 2 column volumes of ET:HX. The ET:HX samples were loaded onto and aspirated through wetted, preconditioned columns, and were

washed twice with 3 ml ET:HX. The ABA fraction was eluted 4 times with 0.5 ml methanol:acetonitrile (1:3,v/v) and the eluate was saved for quantification using ELISA.

Immunoassay Procedure

The ABA was quantified using the monoclonal antibody kit, following the general procedure of Weiler, et al. (1981). A stock solution of 1.0 mM (+/-)ABA standard was prepared in 100% methanol and diluted in Tris buffer, pH 7.5. Concentrations ranging from 0 to 2.0 pmol/100 ul and 100 pmol/100 ul (non-specific binding) were used to form the standard curve. Tissue sample eluates were serial-diluted in Tris buffer, pH 7.5, to 1/1000. Strips of flat-bottomed reaction wells coated with ABA McAB were placed in strip holder plates. To each reaction well, 100 ul of either diluted tissue sample or ABA standard was added. Following addition of the standards or samples, 100 ul of ABA-alkaline phosphatase conjugate in Tris buffered saline was added to each well. The holder plates were sealed and placed in the dark at 4 C for 3 h. After incubation, solutions were decanted and wells were washed 3 times with a tween-saline solution. After washing, enzyme tracer activity was assayed by the addition of p-Nitrophenyl phosphate substrate (200 ul) to each well. Substrate, wash solution, and enzyme tracer were each added using a 4-channel micropipettor (Titertek). Holder plates were subsequently incubated at 37 C in a forced air microtitration plate oven (Titertek, Flow Labs) for 60 min. At the end of the incubation period strips were removed and 1 drop of 1 N NaOH was immediately added to each well to stop the reaction. Activity in each well was read using a Titertek miniskan vertical path spectophotometer (Flow Labs) at 405 nm. All standards and

samples were replicated three times.

Quantification and Validation of the Assay

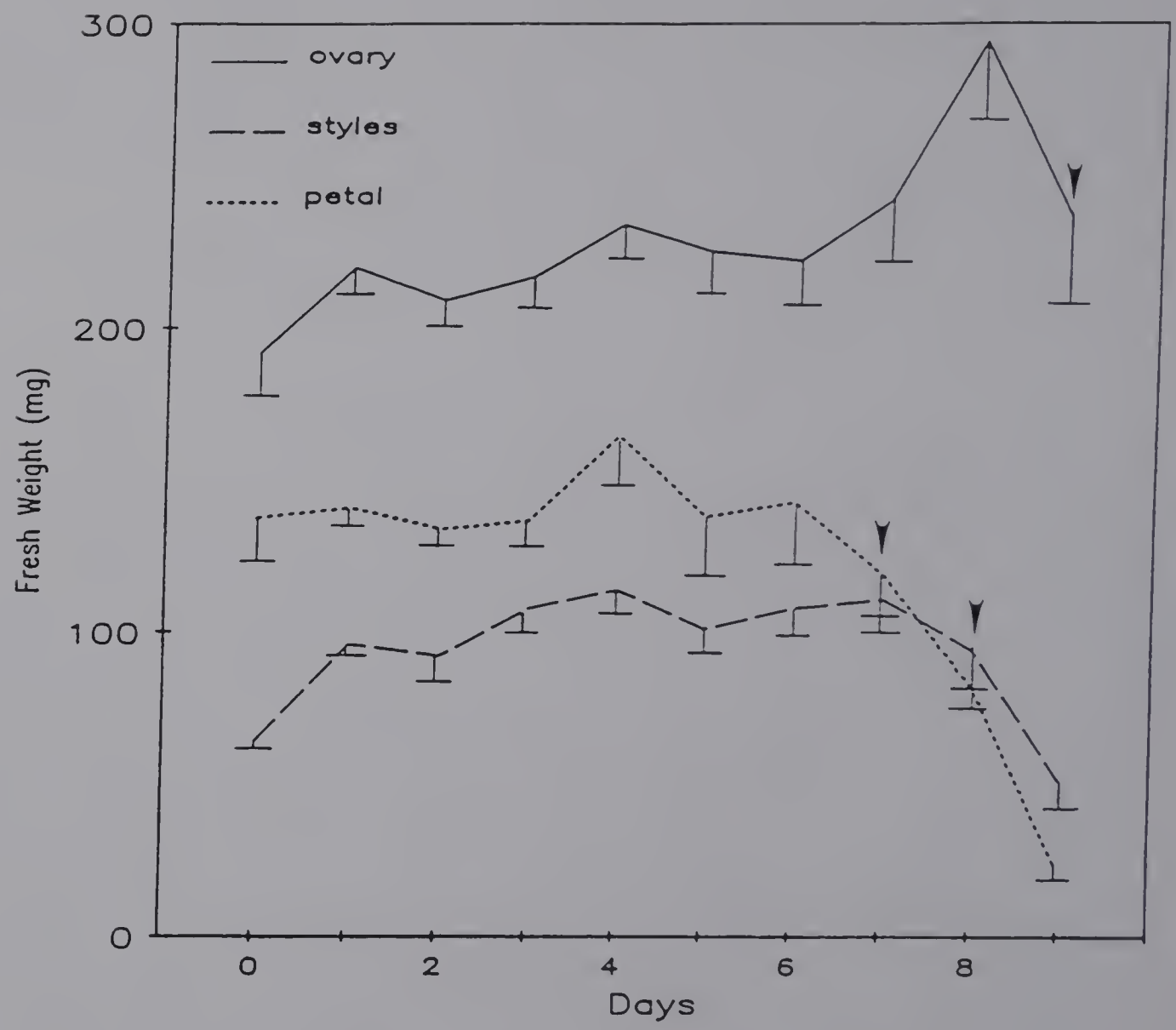
Absorbance readings for the (+/-)ABA standards were converted to percent binding (B/B_0 %) and then logit transformed (Weiler, et al., 1981), resulting in a linear standard curve. The racemic ABA used to form the standard curve contains 50 % of each of the enantiomorphs, R and S-ABA. The monoclonal antibody is raised against S-(+)ABA, and does not bind to R-(-)ABA, allowing use of the racemic ABA with no distortion of the standard curve (Mertens, et al., 1983, Walton, et al., 1979). All sample absorbance readings were converted to B/B_0 %, logit transformed, ABA concentration was extrapolated from the linear regression line of the (+/-)ABA standard curve, and amounts were halved to equal only quantities of physiologically active S-(+)ABA. To determine whether there was any interference in the assay from the sample extracts, replicate samples of petal extracts were added to increasing concentrations of ABA standards (Walker-Simmons, 1987). ABA standards alone and ABA standards plus extract were assayed for ABA content as outlined above and contents plotted as ABA added versus ABA found. Any interfering substances in the petal extract should change the slope of the curve (Pengelly, 1977). The degree of parallelism between lines was evalulated.

Results

Fresh Weight and Visible Turgidity of Flower Parts

At 22 C the fresh weight of the petals remained fairly constant through 6 days postharvest, after which it decreased rapidly throughout the senescing period (Fig. 11). Petal wilting was recognizable at 7 days. The fresh weight of the styles increased from days 0 to 4,

Figure 11. Fresh weights of ageing carnation flower parts harvested (cut) after day 0 to 9 days at 22 C in continuous light. Arrows indicate first visible sign of wilting in each part. Each value represents the mean (n=3). SE bars are indicated.


300
200
100
0
ovary
styles
petal
Fresh Weight (mg)
0
2
4
6
8
Days

remained fairly constant from days 4 to 7, and then decreased. Visible loss of turgidity was observed at 8 d. Fresh weight of the ovary increased from day 6 to day 8, and then decreased at 9 days as it lost turigidity (Fig. 11). Apparently, neither the receptacle nor the green tissue senesced with the remainder of the flower, there being no significant decrease of fresh weight or visible loss of turgidity of either part as the flower aged and senesced (data not shown).

Percent Recovery of [^{14}C]ABA after Purification Procedures

When radiolabeled ABA was added to samples of styles, ovaries, and green tissue as an internal standard, there was no significant difference in percent recovery between any of the three plant parts. After partitioning, 89 to 91 % of the label was recovered, and after silica gel chromatography 55 to 77 % was recovered. At both steps, the differences among tissues were within the standard errors (n=3).

Cross Reactivity of Antibody

The suppliers of the ABA McAb report (Phytodetek, personal communication) that it has cross reactivities of 100 % with 2-cis-(S)-ABA. Less than 0.1 % cross-reacts with either 2-cis-(S)-ABA or the ABA metabolites, phaseic acid and dihydrophaseic acid. They report no cross reaction with either 2-cis-(R)-ABA, 2-trans-(S)-ABA, ABA glucopyranosyl ester and -cis-diol, xanthoxin, or any trans-farnesol.

Standard Curve and Validation of the ABA Immunoassay

A linear standard curve was constructed from data points for 0.05 to 2.0 pM per assay (+/-)ABA (appendix, Fig. 20). The sensitivity of the assay is optimum between 0.02 and 5.0 pM (Phytodetek, personal communication). The addition of purified petal extract did not interfere

with the activity of the immunoassay procedure. Plotting the ABA added vs. ABA found for both ABA standard and ABA standard + extract resulted in parallelism between lines (appendix, Fig. 21).

Levels of ABA and Ethylene

A significant increase in the total activity of ABA per flower part in the green tissue and receptacle was detected after 6 and 7 days, respectively (Fig. 12). Levels decreased substantially in the green tissue on day 9, while in the receptacle ABA continued to accumulate on day 9. When levels of ABA were calculated on a fresh weight basis, there was almost a 7-fold increase from 0 to 9 d in the receptacle, but mostly during days 7 to 9, versus less than a 2-fold increase in the green tissue during days 0 to 8 (Fig. 13). In the petals a small, transient increase of ABA was observed at 3 and 4 days, followed by a large increase of after 5 days (Fig. 14). The ethylene climacteric in the petals corresponded with the ABA increase after 5 days (Fig. 14). By 9 days both ABA levels and ethylene production had returned to presenescent levels.

A modest peak of ABA activity was detected in developing styles at 2 d, with a decrease at 3 d and a subsequent increase after 4 d (Fig. 15). Levels of ABA in the ovary started increasing after 3 d. Beginning at 5 d, and continuing through 9 d, the levels of ABA in both the styles and the ovary paralleled each other, with large peaks observed at 7 d and high levels remaining at 9 days. The styles contained the highest amount of ABA of any flower part when calculated on a fresh weight basis, at approximately 700 ng/ g FW (Fig. 13).

Figure 12. ABA content of the receptacle and green tissue parts from carnation flowers harvested after 0 to 9 days at 22 C and continuous light. Arrow indicates the ethylene climacteric peak in the petals. Each value represents the mean (n=3). Standard error bars are indicated.

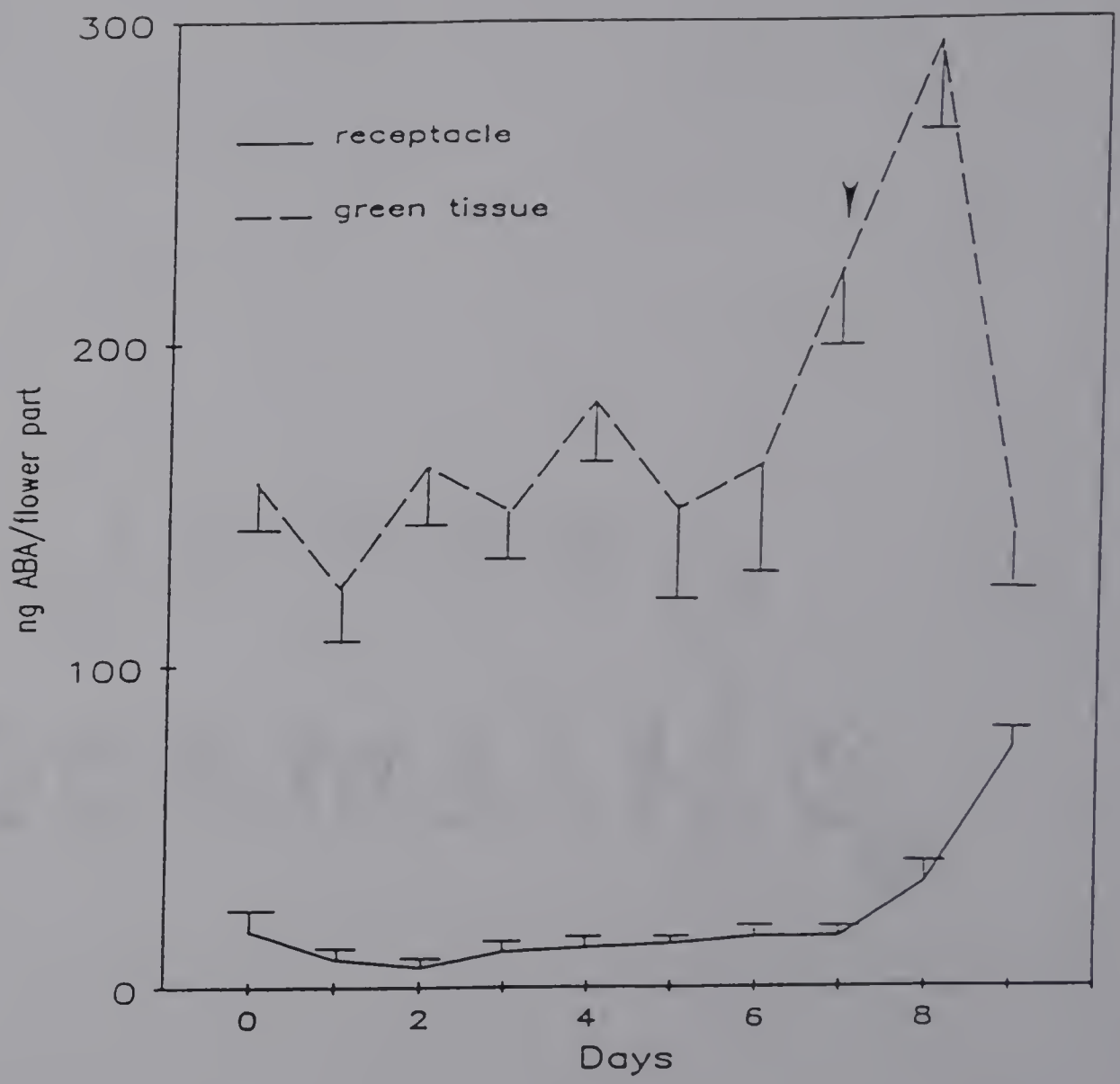

300
200
100
0
ng ABA/flower part
receptacle
green tissue
0
2
4
6
8
Days

Figure 13. ABA content per gram fresh weight of excised carnation flower parts harvested from flowers after 0 to 9 days at 22 C and continuous light. Arrow indicates the ethylene climacteric peak in the petals. Each value represents the mean (n=3). Standard error bars are indicated.

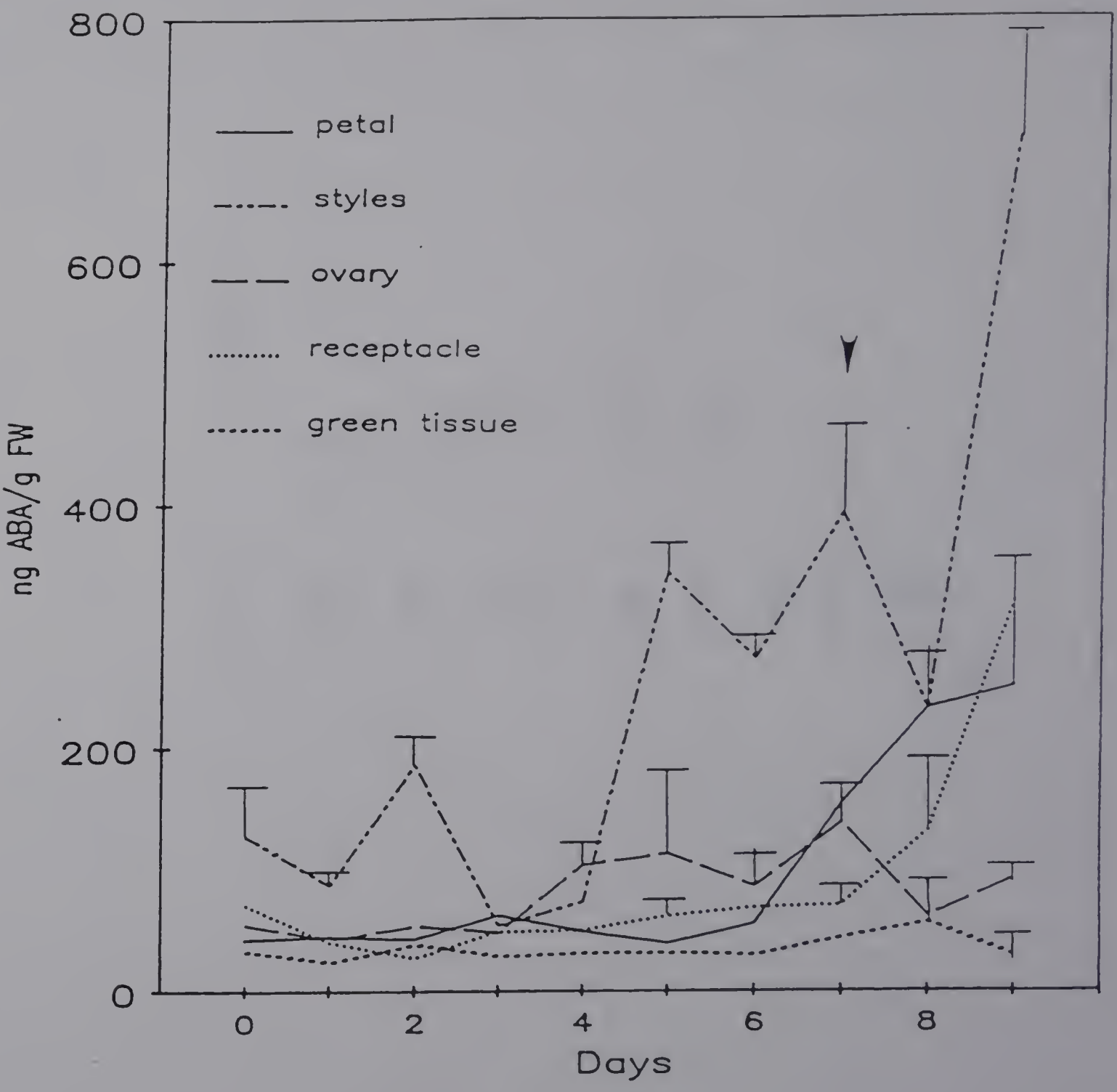

800
600
400
200
0
ng ABA/g FW
petal
styles
ovary
receptacle
green tissue
0
2
4
6
8
Days

Figure 14. The ethylene production and ABA content of excised carnation petals harvested from flowers after 0 to 9 days at 22 C in continuous light. Arrow indicates first visible sign of wilting. Each value represents the mean (n=3). Standard error bars are indicated.

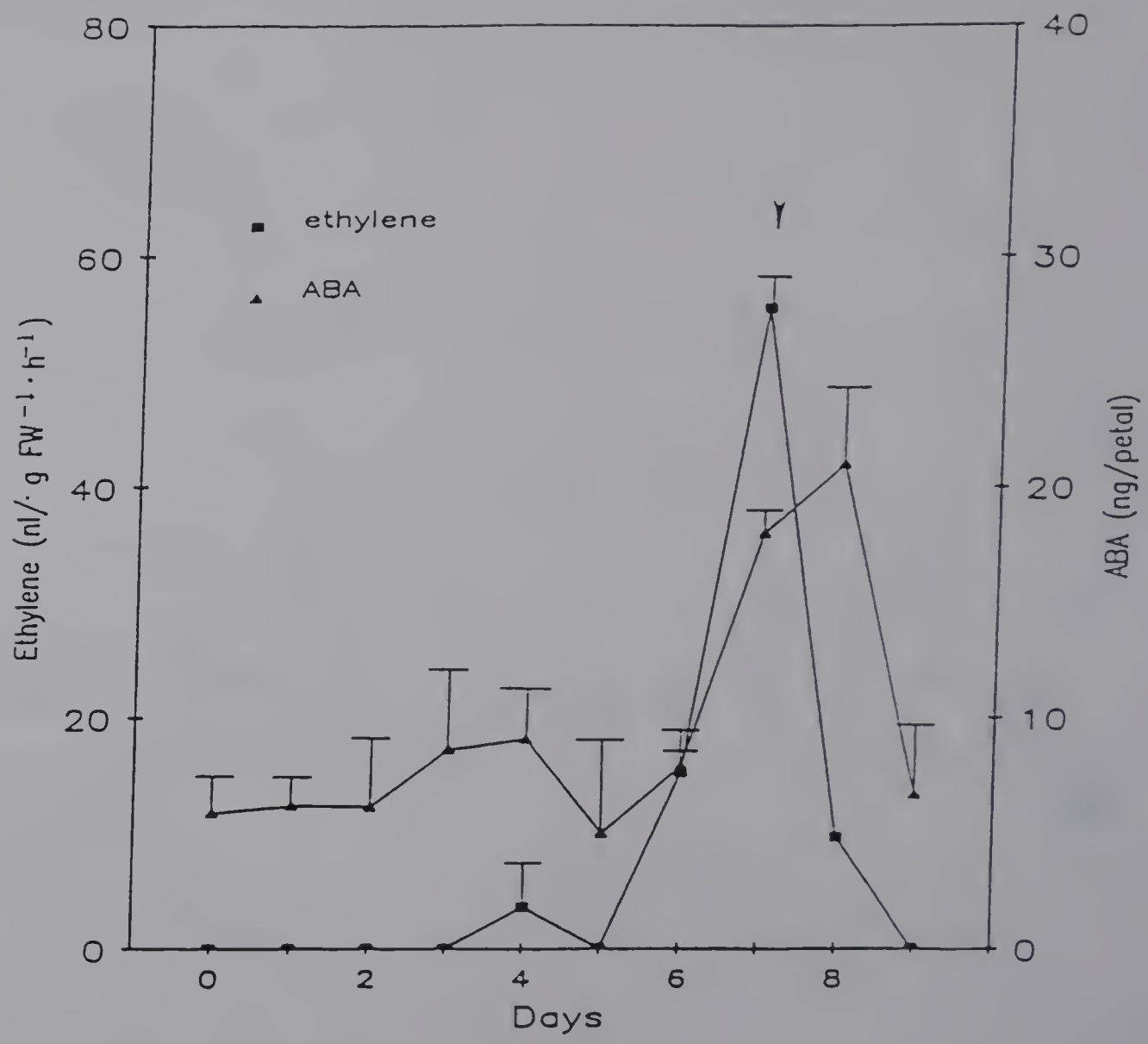
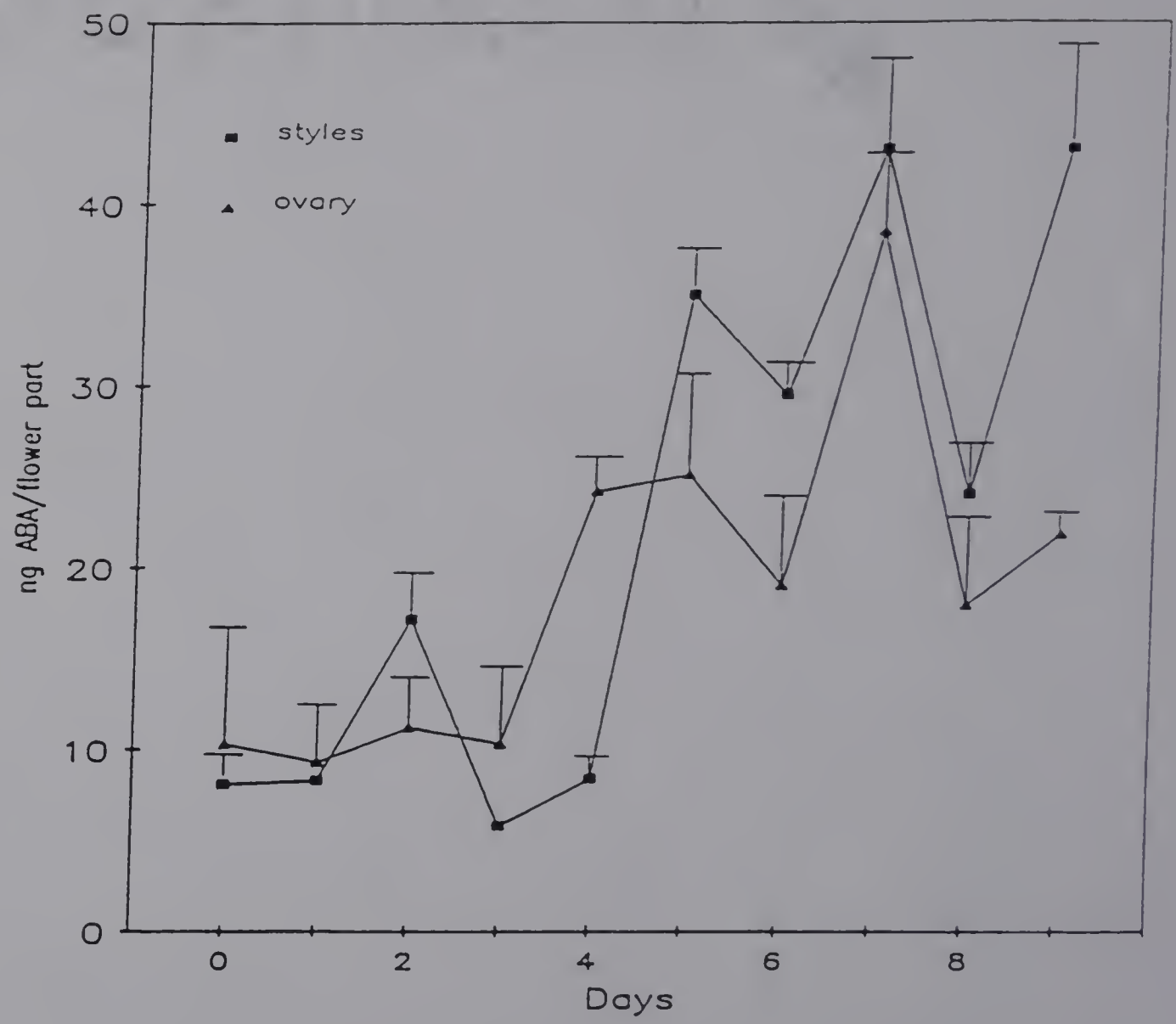

80
60
40
20
0
40
30
20
10
0
ethylene
ABA
Ethylene (nl/· g FW⁻¹·h⁻¹)
ABA (ng/petal)
0
2
4
6
8
Days

Figure 15. The ABA content of the styles and the ovary from carnation flowers harvested after 0 to 9 days at 22 C under continuous light. Each value represents the mean (n=3). Standard error bars are indicated.


50
40
30
20
10
0
styles
ovary
ng ABA/flower part
0
2
4
6
8
Days

Discussion

From the time a carnation flower first opens and is harvested, to when it ultimately senescences, many morphological and biochemical changes occur. The morphological changes include an elongation of the styles, enlargement of the petals (Manning, 1981), and swelling of the ovary (Mor, et al., 1980). Without fertilization these organs will die, leaving behind the chlorophyllous parts of the flower seemingly unaffected. Carnation petal wilting, coincident with the ethylene climacteric, is very distinct and reproducible, making it an excellent marker to use in studying the biochemical changes associated with the onset of senescence.

Abscisic acid is thought to play a major role in the regulation of flower senescence (Mayak and Halevy, 1981). Previous studies have tried to correlate the endogenous levels of ABA and the increase of ethylene production (Eze, et al., 1986, Harris and Dugger, 1986, Nowak and Veen, 1982). The synchronous behavior of these two hormones in senescing tissue has made it very difficult to assess their roles with the physiological event. In this study we used an enzyme immunoassay that enabled us to quantify ABA levels in small amounts of tissue. In previous studies ABA in ageing carnation flowers was quantified using electron capture-gas liquid chromatography (EC-GLC) following methylation (Eze, et al., 1986, Nowak and Veen, 1982). In this investigation a small, temporary increase of ABA was noted in the preclimacteric petals (Figure 14). Eze, et al. (1986), did not detect an increase of ABA in ageing carnation petals until the onset of petal wilting, when vast amounts of ABA were being produced. When comparing

the immunoassay method used in this investigation to EC-GLC used in another study (Nowak and Veen, 1982), quantities of ABA measured for different carnation flower parts were very similar. It appears that the immunoassay may be a more accurate means of quantification due the level of sensitivity of the assay.

In this investigation, levels of ABA in both the petals and the green tissue paralleled the ethylene climacteric rise in the petals (Figs. 12 and 14). A similar coupling of ABA and ethylene levels is evident in some ripening fruit, and it is thought that increased ABA content may be involved in the ripening process once ripening is initiated (Rhodes, 1981). This involvement may be related to the de novo synthesis of degradative enzymes induced by ABA (HO, 1983). A similar mechanism may be involved in flower senescence. The synthesis of degradative enzymes, including RNAase, DNAse, and ATPase, have been detected in various flower types with the onset of senescence (Adam, et al., 1983, Matile and Winkenbach, 1971, Trippi and Paulin, 1984).

The ABA content in the receptacle also increased with petal wilting, but not until after the rise in ethylene (Fig 12). An increase in ABA content is frequently associated with water stress in the tissue (Davies and Mansfield, 1983). Although neither the green tissue nor the receptacle seemed to be under water stress, they both appeared to be influenced by wilting in the petals, which began at 7 days. The styles and the ovary began wilting at 8 and 9 days, respectively (Fig. 11). Given the anatomical proximity of the receptacle to the other parts of the flower, perhaps it continues to accumulate ABA longer than the green tissue in response to wilting in the styles and ovary as well as the

petals.

If ABA content is to be used an indicator of ontogeny of carnation flower parts, it is difficult to speculate what role increased levels are having in the presenescent flower. In both the styles and the ovary an initial peak of ABA was observed in the young, developing flower, with a subsequent peak occurring coincident with petal wilting (Fig. 15). A similar increase of ABA was observed in both preclimacteric carnation flower pistils (Nowak and Veen, 1982) and developing citrus flower styles (Goldschmidt, 1980). Although ABA is frequently regarded as a deleterious plant hormone, it is also involved in many promotive physiological events (Ho, 1983). The involvement of ABA in regulating assimilate partitioning (Tietz, et al., 1981) has been proposed as a possible explanation for increased ABA in the developing gynoecium (Nowak and Veen, 1982). In our investigation, though, the increase of fresh weight in the styles occurred prior to the ABA increase (Figs. 11 and 15). In the ovary, an increase of fresh weight was not apparent until several days subsequent to the initial ABA increase (Figs. 11 and 15). The ABA produced in the ovary with the onset of senescence may be involved in assimilate partitioning, as a dramatic increase of fresh weight was observed at 8 days (Fig. 11).

In this investigation ethylene production in the petals peaked at 7 days after opening (Fig. 14). Previous studies have shown synchronous ethylene climacteric peaks in all the individual parts of the carnation flower (Hanley, et al., 1988). The increase of ABA in the styles and ovary, prior to any increase of ethylene, indicates a separation of the relationship between these two hormones. This separation may be related

to the pattern of senescence in pollinated and unpollinated carnation flowers. Unpollinated, fully open carnation flowers begin to senesce five to seven days after harvest (Hanley, et al., 1988, Nichols, 1977), while wilting in pollinated flowers commences in two to three days (Nichols, 1977, Nichols, et al., 1983). Pollination-induced senescence is mediated by an increase in ethylene production at this time. Manning (1981) found substantial ethylene-forming enzyme activity in young, developing carnation flower styles. It has been suggested that the style may be the triggering organ for pollination-induced flower senescence (Goldschmidt, 1980). Given the causal relationship between ABA and ethylene in senescing tissue (Mayak and Halevy, 1981), the increased production of ABA found in the developing gynoecium may be a necessary component for the onset of pollination-induced senescence.

C H A P T E R V

SUMMARY OF DIFFERENT FACTORS MEDIATING THE SENESCENT RESPONSE IN CARNATION FLOWERS

The pattern of carnation flower senescence observed in this study was very similar to that which has been noted previously (Bufler, et al., 1980, Nichols, 1971). A climacteric rise in ethylene resulted in the onset of irreversible wilting which was first visible in the petals. The general pattern of senescence is very similar in both unpollinated and pollinated carnation flowers, with the latter occurring 3 to 4 days sooner than the former (Nichols, 1977). Both sequences of senescence appear to be triggered by increased ethylene production.

Different parameters of senescence, including ACC and ABA concentrations, increased concurrently with the ethylene surge. In addition to the changes observed at the onset of senescence, I also detected significant activity of different factors influencing the senescent response occurring in the preclimacteric flower. The appearance of both ABA and MACC in the young carnation flowers leads to my speculating on the role of these potential modulators of ethylene biosynthesis prior to the onset of senescence.

The data presented in these series of experiments support the hypothesis that the significant biochemical changes occurring in the preclimacteric flower may be involved in mediating the onset of accelerated pollination-induced senescence. To summarize this theory I have divided my study of the ageing carnation flower into two parts:

1) Endogenous factors relating to the onset of senescence
2) Exogenous factors mediating a response

Endogenous Factors

Because the onset of senescence is so intimately allied to the increased production of ethylene, it was important to study the different parameters relating to ethylene biosynthesis. Abscisic acid (ABA) has been causally associated with the promotion of increased ethylene in senescing tissue (see chapter III). In this investigation the increased ABA level in the petal tissue was concurrent with the increase of ethylene. It was not possible to attribute a specific role to either growth substance based the activity in the petals. Similar increases were also detected in the receptacle and the green tissue with the onset of petal wilting. However, increases of ABA were detected in the styles and ovary at 3 and 4 days, respectively, indicating activity of a senescence-promoting growth regulator well before the beginning of any visible deleterious effects in the flower. The increase of endogenous ABA activity may be critically involved in the triggering mechanism inducing the onset of accelerated senescence upon pollination. Given the involvement of ABA in the induction of ethylene synthesis, the appearance of the growth regulator in the styles and ovary may be necessary for increased ethylene production following pollination.

The significant increases of endogenous 1-aminocyclopropane-1-carboxylic acid (ACC), observed just prior to or concomitant with the increase of ethylene, is in agreement with earlier studies (Hsieh and Sacalis, 1986, Whitehead, et al., 1984a). Upon pollination, a similar increase of ACC is induced in the young carnation flower (Reid, et al., 1984). In this study a large peak of MACC was observed at 3 days after flower opening, and was immediately followed by the degradation of the

metabolite at 4 days, with no detectable increase of ACC or ethylene. This is the first report of a significant change in the concentration of MACC, the malonyl conjugate form of ACC, occurring in the preclimacteric flower. Generally, MACC accumulates as an inactive endproduct of the metabolism of ACC to ethylene (Hoffman, et al., 1983). This dramatic turnover of the conjugate form of a potential ethylene precursor lends additional plausibility to the hypothesis that the activity of MACC detected in the preclimacteric flower may be involved in mediating pollination-induced senescence. Because the stylar tissue contains significant ethylene-forming enzyme (EFE) activity (Manning, 1981), it seems reasonable that a potential pool of ACC would be found in a conjugated, inactive form. If metabolized to ACC, an immediate burst of ethylene production should occur. Other plant tissues have been found to have the enzymatic ability to hydrolyse MACC to ACC and produce ethylene (Jiao, et al., 1986).

Exogenous Factors

For the accumulation of endogenous MACC to be involved in the biosynthesis of ethylene, the preclimacteric carnation flower petals must possess the enzymatic ability to hydrolyze the metabolite to ACC. When excised, 3-day-old carnation petals were treated with exogenous MACC, ethylene production and subsequent wilting was induced, but not until a lag of approximately 33 hours (Hanley, et al., 1988). As was shown in previous studies involving vegetative tissues (Jiao, et al., 1986), the MACC-induced ethylene production is dependent on both time and substrate-concentration. The MACC-induced ethylene production by the petals was

attributed to the hydrolysis of MACC to ACC and not the de novo synthesis of ACC in the tissue (see chapter II). This finding supports the hypothesis that endogenous MACC in the preclimacteric petals is a potential source of ACC and ethylene evolution.

The dramatic decrease of endogenous MACC at 4 days did not result in any detectable ACC or ethylene. In this investigation I observed the metabolism of ACC in the preclimacteric petals to products other than ethylene after administering radiolabelled ACC and incubating 24 h (see Chapter III). Most of the exogenous ACC was metabolized, with very little recovered as free ACC, similar to the endogenous reaction. A plausible position would be that the supply of endogenous MACC present in tissue is used as an ethylene source upon pollination, but if the flower is not pollinated no accelerated senescence transpires, and the MACC is rapidly turned over in other metabolic pathways. An outline of the possible reaction mechanism involved in the turnover of MACC is detailed in figure 22 of the appendix.

The studies included in this dissertation were initiated in hopes of elucidating the factors involved in the triggering of ethylene production at the onset of senescence. The distinct biochemical changes observed in the preclimacteric flower may be involved in regulating the ethylene evolution, and should be used in further studies as it relates to early onset of senescence-induced pollination.

A P P E N D I X A

Equations for Conversion of Enzyme Immunoassay Absorbance Readings

1) Percent binding (B/B_0 %)-

$$\frac{\text{standard or sample O.D. reading - NSB O.D. reading}}{B_0 \text{ O.D. reading - NSB O.D.}} \times 100$$

O.D.= optical density
NSB = non-specific binding
B_0 = buffer + tracer = 100 % binding

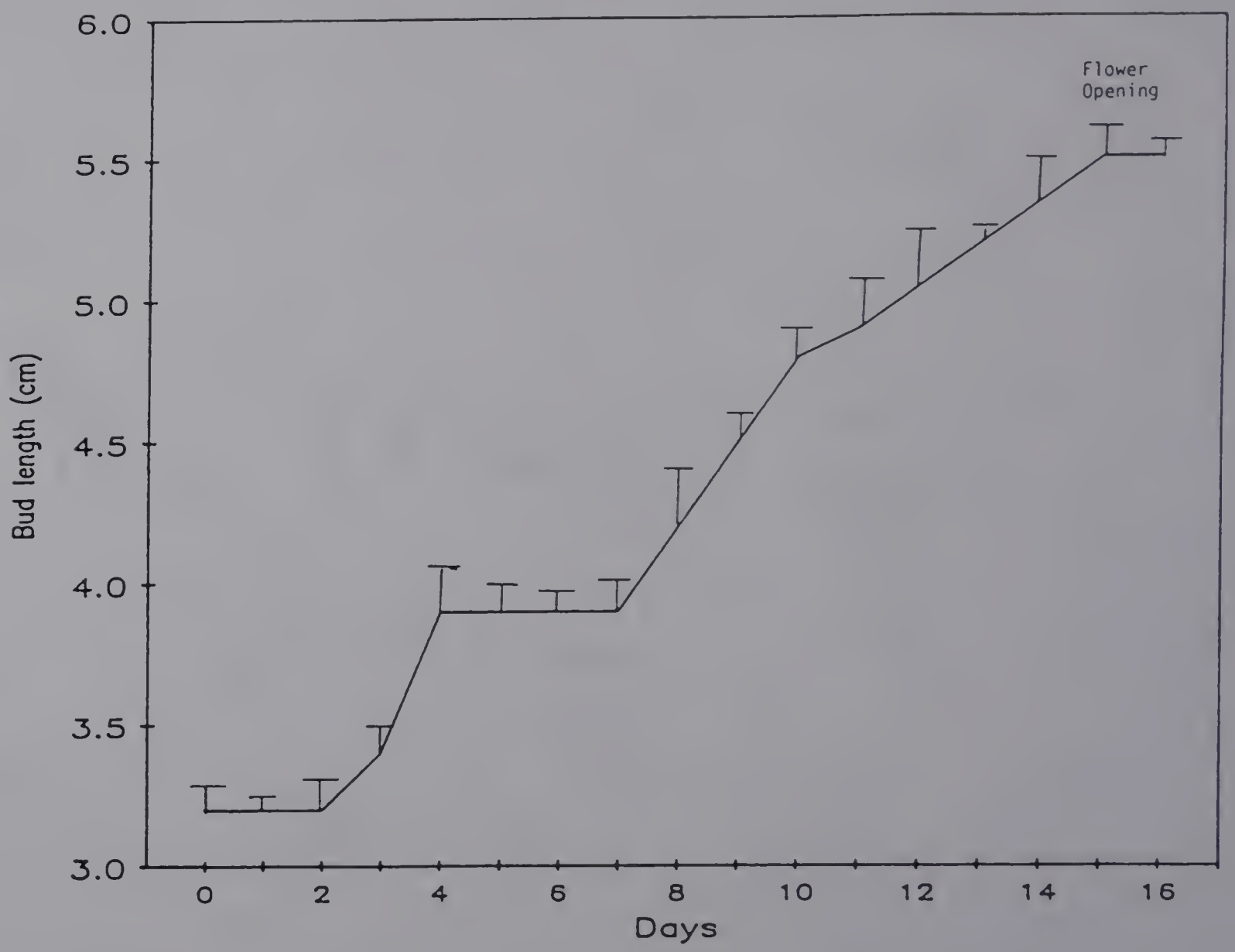
2) Logit transformation

$$\text{Logit } B/B_0 = \text{Ln } (B/B_0 \text{ \%} / 100\text{-}B/B_0 \text{ \%})$$

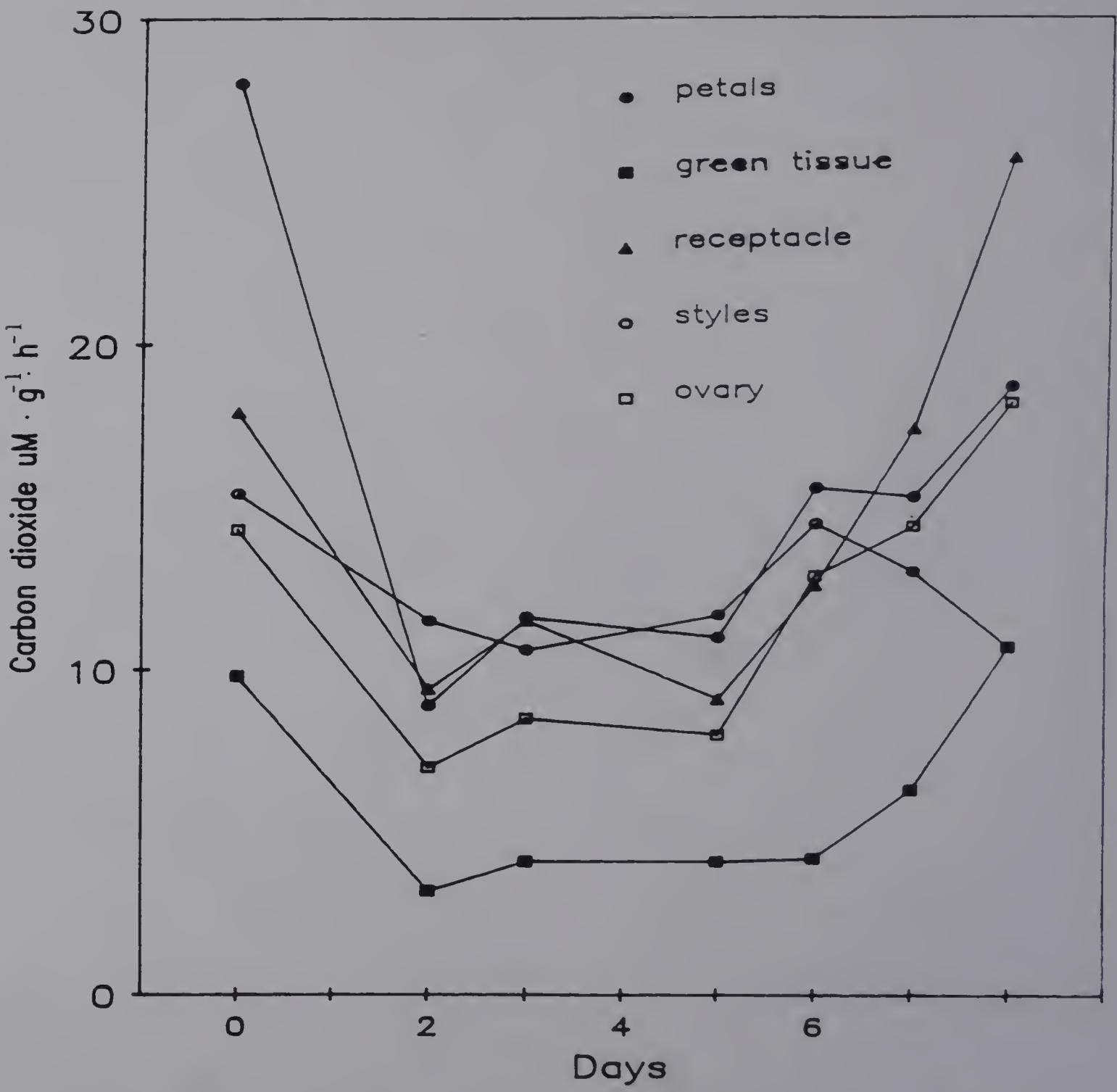
A P P E N D I X B

Figures

Figure 16. Days to carnation flower opening as observed after following flower bud growth. Flowers were first fully open after attaining a bud length of 5.5 cm. Standard error bars are indicated (n=12).


6.0
5.5
5.0
4.5
4.0
3.5
3.0
Bud length (cm)
Flower
Opening
0
2
4
6
8
10
12
14
16
Days

Figures 17. Production of carbon dioxide of excised carnation flower parts from flowers harvested (cut) at 0 days and aged 0 to 9 days at 22 C in the dark. Data points are the mean of two replications.


30
20
10
0
Carbon dioxide uM · g⁻¹ · h⁻¹
petals
green tissue
receptacle
styles
ovary
0
2
4
6
Days

Figures 18. Production of ethylene of excised carnation flower parts from flowers harvested (cut) at 0 days and aged 0 to 9 days at 22 C in the dark. Data points are the mean of two replications.

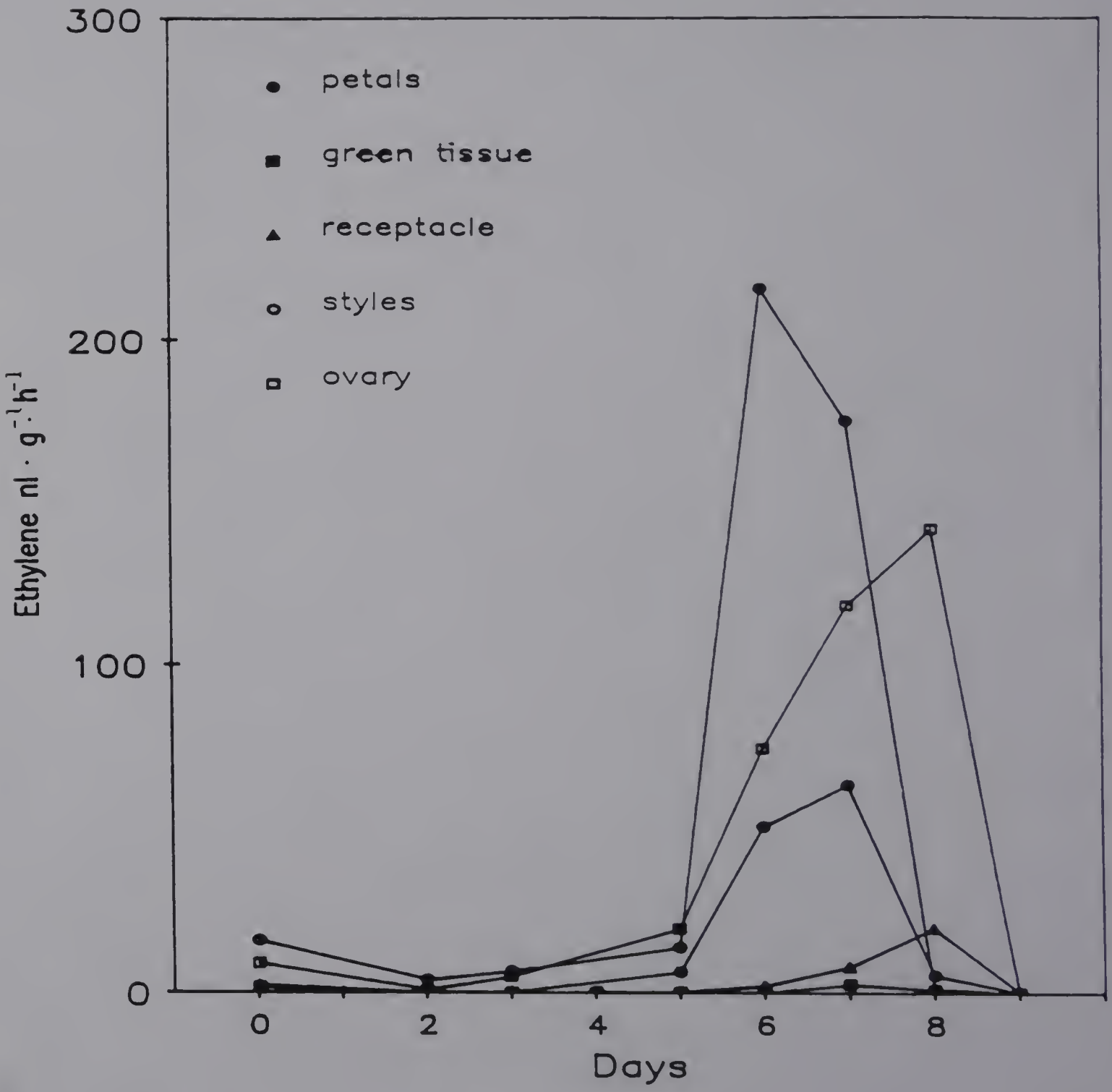

300
200
100
0
petals
green tissue
receptacle
styles
ovary
Ethylene nl · g⁻¹·h⁻¹
0
2
4
6
8
Days

Figure 19. Distribution of radiolabelled metabolites of [^{14}C]MACC in 3- and 4-day-old preclimacteric carnation petals after incubating in the light 24 h. Labeled MACC was recovered from intact carnation petals that were administered labeled ACC, and extracted and purified as outlined in chapter III. Authentic [^{14}]ACC and MACC were detected at Rfs 0.2 and 0.5-0.6, respesctively, on the thin-layer plate chromatogram. Data values are the mean of two replications.

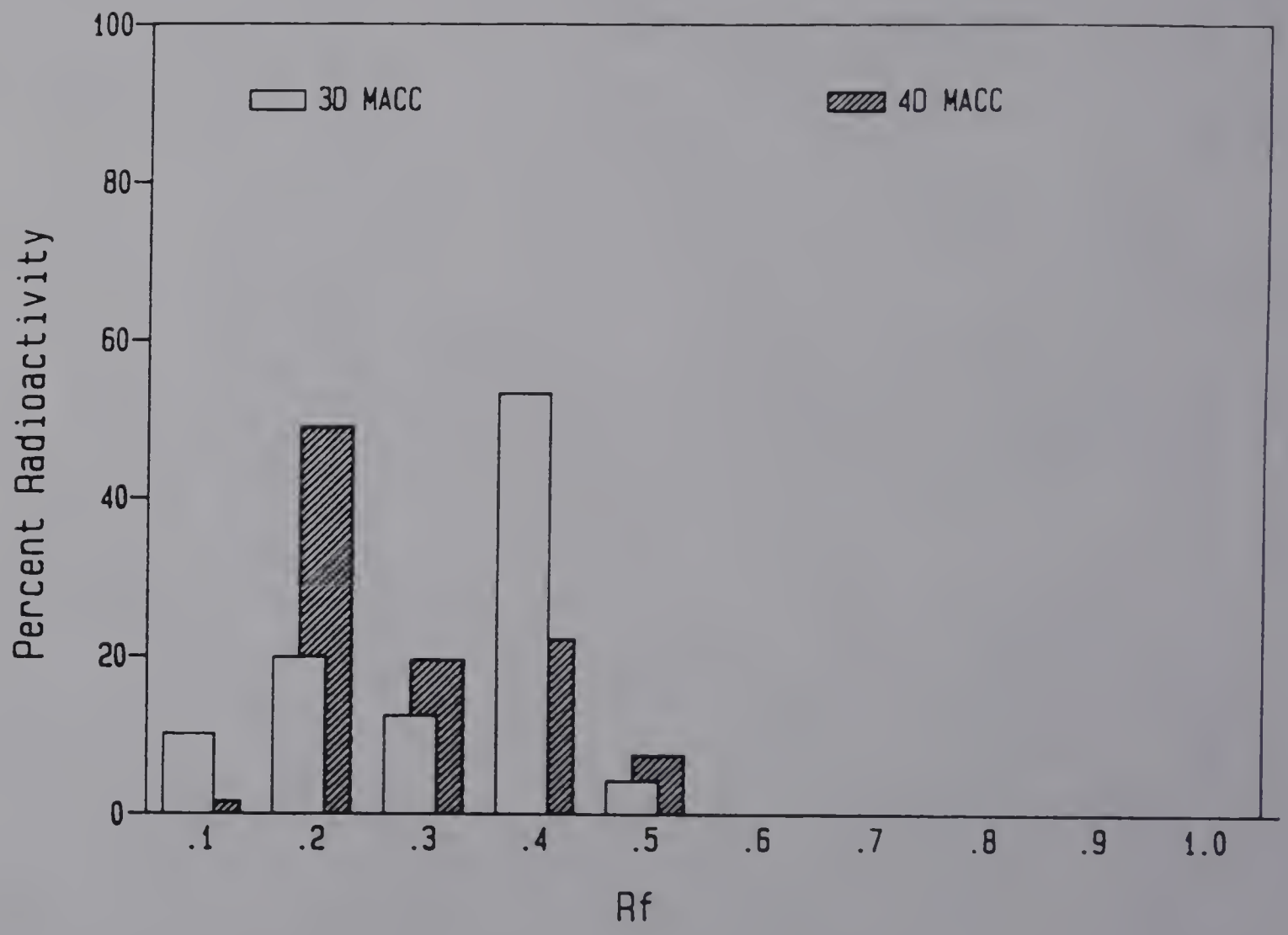

100
80
60
40
20
0
3D MACC
4D MACC
Percent Radioactivity
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
Rf

Figure 20. ABA enzyme immunoassay standard curve. Data values are the mean of three replications. Linear regression line for data is indicated.

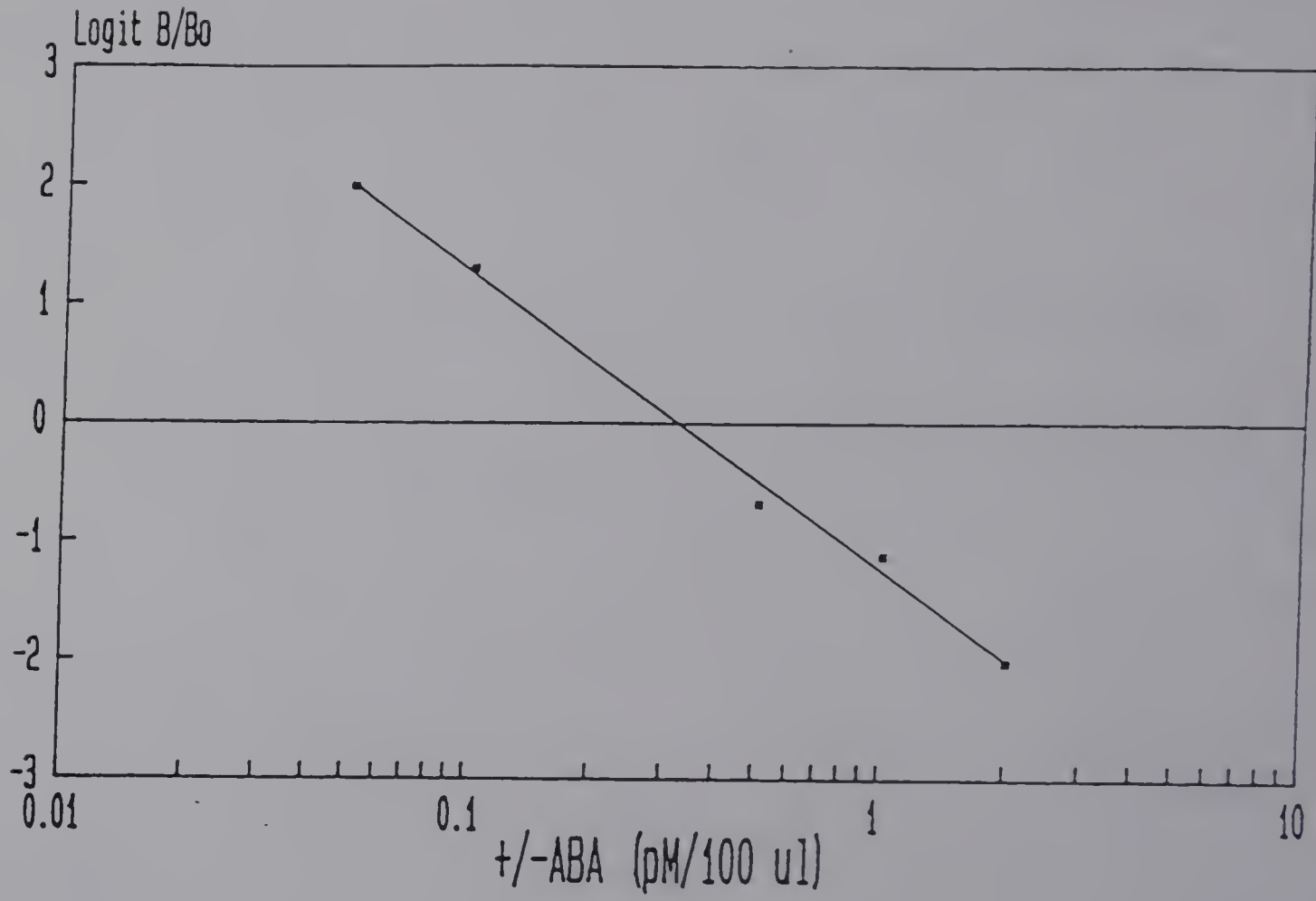
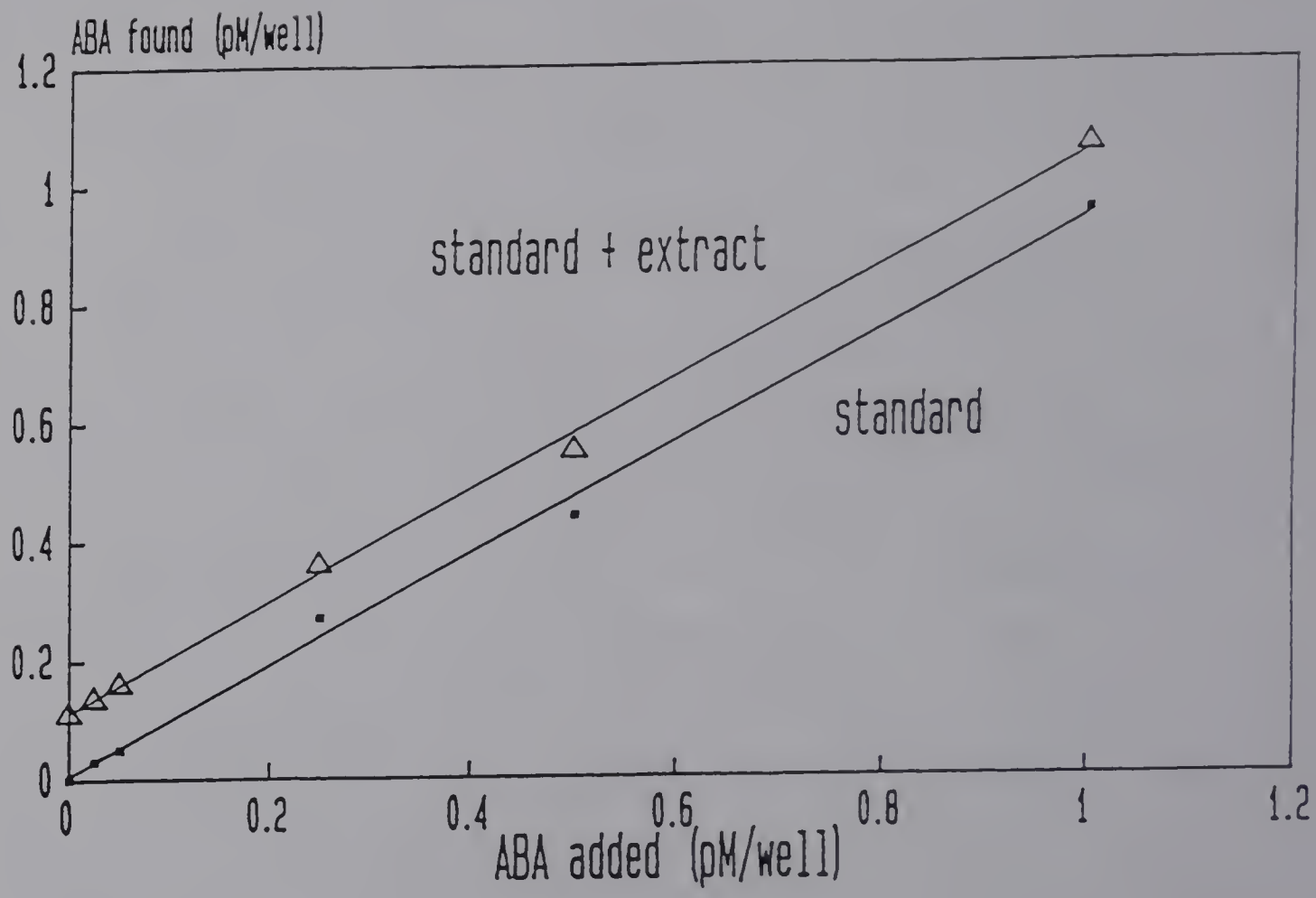

Logit B/Bo
3
2
1
0
-1
-2
-3
0.01
0.1
1
10
+/-ABA (pM/100 ul)

Figure 21. Validation of ABA enzyme immunoassay. Data values are the mean of three replications. Linear regression line for data is indicated.


ABA found (pM/well)
1.2
1
0.8
0.6
0.4
0.2
0
standard + extract
standard
0
0.2
0.4
0.6
0.8
1
1.2
ABA added (pM/well)

A P P E N D I X C

Proposed Mechanism of the Activity of MACC in Ageing Carnation Flower Petals.

The following outline is a proposed mechanism for the activity of MACC in ageing carnation flower parts. This outline follows the schematic pathway as represented in figure 22.

The steps are as follows:

1) The malonylation of ACC to MACC from after day 1 to day 3.

2) Day 3- peak MACC levels induce the activity of an aminoacylase (Matern, et al., 1986). This hydrolysis is both concentration and time-dependent (Hanley, et al., 1988, Jiao, et al., 1986).

3) Little to no free ACC or ethylene is detected in preclimacteric carnation petals (Bufler, et al., 1980, Hanley, et al., 1988). Young petals do not possess active ethylene-forming enzyme (EFE) (Manning, 1981) activity to generate ethylene from ACC. The free amino acid would be degraded by decarboxylation and/or transaminase activity (Zubay, 1986) and subsequent ring-opening (Yang, 1985), resulting in a 2-3 carbon intermediate. Products of ACC catabolism could enter the shikimate acid pathway as pyruvate or phosphoenolpuruvate.

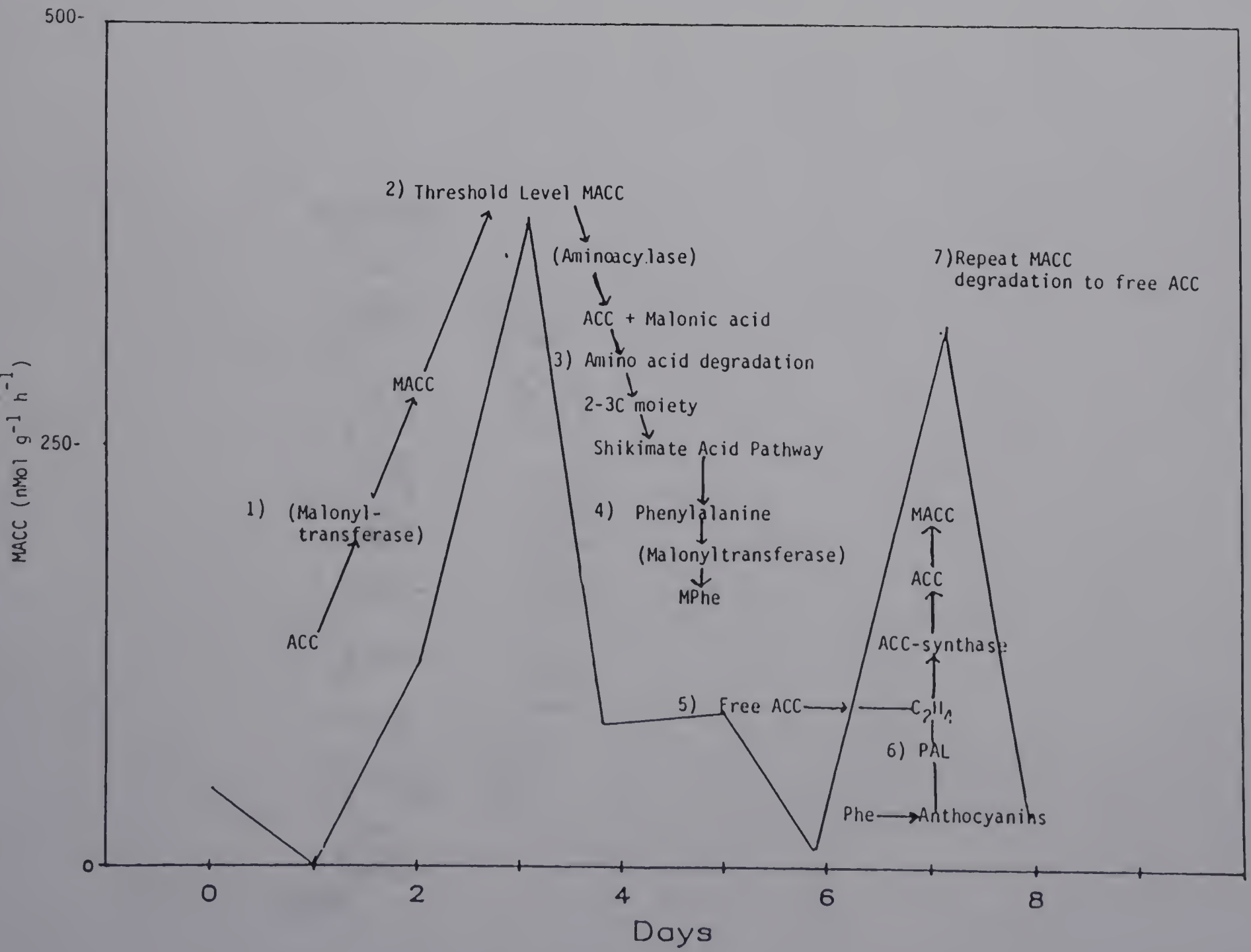
4) L-phenylalanine (Phe) is a major product of the shikimate acid pathway (Goodwin and Mercer, 1983). At this juncture L-Phe might be acted upon by a racemase enzyme to form D-Phe. D-Phe is a competitive inhibitor of the malonylation of ACC resulting conjugated Phe (Kionka and Amrhein, 1984, Liu, et al., 1983, Liu, et al., 1984) and the same malonyltransferase enzyme conjugates both ACC and Phe (Su, et al., 1985). Consequently, at this time in development very little MACC is detected, and MPhe may be accumulating.

5) An increase of free ACC is detected at 6 days (Hanley, et al., 1988) with the commencement of ethylene production. Increased ethylene induces ACC-synthase activity (Yang, 1985), resulting in a saturating amount of free ACC. ACC substrate not utilised by the EFE in ethylene production is conjugated (Hoffman, et al., 1983), thus a another peak of MACC at 7 days.

6) Increased ethylene production stimulates activity of the phenylalanine ammonia-lyase enzyme (Blankenship and Unrath, 1988) synthesizing anthocyanins using Phe as an intermediate. A large increase of endogenous anthocyanins were detected coincident with the ethylene climacteric peak in carnation petals (see chapter IV). The Phe that had been shunted to MPhe formation may now be utilized for pigment formation and not the malonyltransferase enzyme (thus, also the increase of MACC detected at 7 days).

7) Peak MACC levels at 7 day induces the hydrolysis of MACC to ACC. Coincident with the ethylene climacteric is the onset of irreversible wilting and the loss of an intact membrane. The EFE requires an intact membrane in order to continue generating ethylene (Yang, 1985), thus degradation of ACC as stated above, and not oxidation to ethylene.

Figure 22. A mechanism for the activity of MACC in carnation flower petals.


500-
250-
0
MACC (nMol g-1 h-1)
2) Threshold Level MACC
(Aminoacylase)
ACC + Malonic acid
3) Amino acid degradation
2-3C moiety
Shikimate Acid Pathway
4) Phenylalanine
(Malonyltransferase)
MPhe
MACC
1) (Malonyl-transferase)
ACC
7)Repeat MACC degradation to free ACC
MACC
ACC
ACC-synthase
5) Free ACC
C2H4
6) PAL
Phe
Anthocyanins
0
2
4
6
8
Days

B I B L I O G R A P H Y

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