Initial events and inter-organ relations during senescence of orchid (*Cymbidium*) flowers

Initiële processen en relaties tussen de verschillende bloemdelen tijdens de veroudering van (*Cymbidium*) orchideeën

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Initial events and inter-organ relations during senescence of orchid (*Cymbidium*) flowers

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dr. H. C. van der Plas,
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Stellingen

1. De door emasculatie geïnduceerde verwelking van *Cymbidium*-orchideeën wordt veroorzaakt door uitdroging van het rostellum en niet, zoals eerder gesteld, door mechanische beschadiging of door het wegvallen van de cytokininestroom uit de pollinaria en de meeldraadkap.
   Arditti J and Flick BH, Amer J Bot 61:643-651 (1974)
   Van Staden J, Scientia Hortic 10:277-284 (1979)
   Dit proefschrift

2. Relatief geringe veranderingen in ethyleenproduktie kunnen belangrijke fysiologische effecten bewerkstelligen. De door *Trewavas* geponeerde theorie dat hormoonwerking in planten juist niet via concentratie- maar via gevoeligheidsveranderingen tot stand komt, gaat hier volledig aan voorbij en is daarom tenminste gedeeltelijk onjuist.
   Trewavas AJ, Physiol Plant 55:60-72(1982)
   Dit proefschrift

3. Ethyleenproduktiemetingen aan geïsoleerde plantedelen kunnen, ook als er geen sprake is van een wond-effect, een verkeerd beeld van de *in vivo* situatie geven doordat de communicatie tussen de organen verbroken is.
   Dit proefschrift

4. De door *Whitehead* en *Halevy* gevonden ethyleengevoeligheidsfactoren blijken, zelfs in hun eigen onderzoek, deze functie niet uit te oefenen.
   Whitehead CS and Halevy AH, Plant Growth Regul 8:41-54(1989)
   Dit proefschrift

5. De door *Mor et al.* gesuggereerde betrokkenheid van een mobiele ethyleengevoeligheidsfactor bij de verwelking van anjerbloemblad berust op een foutieve interpretatie van de eigen proefgegevens.
   Mor Y, Halevy AH, Spiegelstein H and Mayak S, Physiol Plant 65:196-202(1985)
   Overbeek JHM and Woltering EJ, Physiol Plant (in press)

6. Met inbegrip van een identieke figuur betreffende de ESR spectra van 1,2-dihydroxybenzeen-3,5-disulfonzuur en een mengsel hiervan met een microsomale membraanfractie van anjerpetalen, is een aanzienlijk gedeelte van de door *Droillard et al.* gepresenteerde gegevens een herhaling van het enkele jaren eerder door *Mayak et al.* gepubliceerde materiaal en had in deze vorm dus niet door een zichzelf respecterend wetenschappelijk tijdschrift geaccepteerd mogen worden.
   Droillard MJ, Paulin A and Massot JC, Physiol Plant 71:197-202(1987)
   Mayak S, Legge RL and Thompson JE, Phytochemistry 22:1375-1380(1983)

7. De grote genetische variabiliteit binnen het snijbloemenassortiment biedt de mogelijkheid de door de handel gewenste interne kwaliteit via veredelingstechnieken in te bouwen waardoor het gebruik van milieubelastende chemicaliën in deze produktgroep teruggedrongen kan worden.

8. Intensivering van de voorlichting levert op korte termijn de grootste bijdrage aan de verbetering van de houdbaarheid van bloemisterijprodukten.

9. Voedseladditieven zijn veelal beter onderzocht op hun toxiciteit dan van nature aanwezige componenten.

10. De resultaten van geavanceerd wetenschappelijk onderzoek worden veelal met behulp van volstrekt verouderde apparatuur gepresenteerd.

11. Er zijn tenminste 2632 verschillende manieren om een literatuurreferentielijst samen te stellen. Het is onwetenschappelijk dat vele, zo niet alle, binnen het wetenschappelijke circuit in gebruik zijn.

12. Voor de meeste mensen zal het openbaar vervoer pas acceptabel zijn als de eigen auto kan worden meegenomen.

13. Het feit dat etheen (C_2H_4) door plantenfysiologen nog altijd met de verouderde naam "ethyleen" wordt aangeduid wekt bij niet-vakgenoten vaak nog meer verbazing dan het gegeven dat dit simpele molekuul hormonale activiteit bezit.

14. Alhoewel ethyleen volgens de *Gas Encyclopaedia* lichtelijk slaapverwekkend is, bezorgt het mij menige slapeloze nacht.

Stellingen behorende bij het proefschrift "Initial Events and Inter-Organ Relations during Senescence of Orchid (*Cymbidium*) Flowers" van E.J. Woltering.

Nature will tell you a direct lie
if she can

Charles Darwin

Unfortunately we are always so
anxious to believe it

VOORWOORD

Het onderzoek naar de regulatie van de veroudering in *Cymbidium*bloemen, zoals beschreven in dit proefschrift, werd tussen begin 1987 en eind 1989 uitgevoerd als onderdeel van het onderzoekprogramma van het Sprenger Instituut en in samenwerking met de vakgroep Plantenfysiologie van de Landbouwuniversiteit.

In de loop van het onderzoek ben ik regelmatig geconfronteerd met toespelingen op de wat vreemde keuze van het proefmateriaal. De afgesneden *Cymbidium*bloem is van nature betrekkelijk lang houdbaar en praktisch/commercieel gezien zijn er dan ook geen grote problemen te verwachten. Onderzoek naar de verouderingsprocessen in deze bloemsoort lijkt daarom het doel voorbij te streven.

Om verschillende redenen is indertijd toch voor deze bloemsoort als onderzoekobject gekozen. Met name het gegeven dat verwijdering van de stuifmeelklompjes en de meeldraadkap (emasculatie) het verouderingsproces - en de daarmee gepaard gaande roodverkleuring van de bloemlip - sterk versnelt, heeft hiertoe bijgedragen. Omdat de verschillende bloemdelen alle vrij fors zijn en ruimtelijk goed van elkaar zijn gescheiden, leek de bloem tevens een geschikt systeem voor de bestudering van de onderlinge relaties tussen de bloemdelen. Het bewijs dat deze visie juist was ligt thans voor u.

De gepresenteerde gegevens bieden diverse aanknopingspunten voor verder onderzoek met deze bloemsoort. Met name de mogelijke aanwezigheid van fysiologisch-actieve celwandfragmenten in het rostellum en de rol hiervan bij de initiatie van de veroudering verdienen nader onderzoek. Hetzelfde geldt voor het mechanisme dat verantwoordelijk is voor het snelle transport van ethyleen en ACC door de bloem.

Om met Prof. dr. Zbygniew Prlwytzkofski te spreken: "Praw! Dezer proefneming is ja op bevredigender wijze verlopen. Nu kan ik mij rustig heenzetten en het waarom des fenomenes studeren. An der arbeit, mijnheer Pieps!"

Ik wil hierbij iedereen bedanken die op een of andere wijze heeft bijgedragen aan het tot stand komen van dit proefschrift. In het bijzonder

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- Margreet Jordens en Rob Bons voor het ver- en bewerken van de ruim 20 concept-teksten (spoed) en al het technische tekenwerk (haastklus);
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- Mijn ouders, omdat ze mij altijd mijn eigen gang lieten gaan.

Ernst Woltering
Arnhem, april 1990

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

In order to successfully survive all living organisms need to have an adequate system to respond to the continuously changing environment and to regulate the various developmental processes in cells and organs at different locations in the body. This chapter briefly discusses the currently recognized regulatory mechanisms in animals and plants, the biosynthesis and mode of action of the gaseous plant hormone ethylene (C_2H_4), and the role of ethylene and other "wilting factors" in the regulation and coordination of senescence in flowers.

Regulatory mechanisms in animals

The mammalian nervous system contains an incredibly complex collection of neurons with many billions of connections between them. Approximately 1 g of cerebral cortical gray matter may contain up to 200 million neurons and each of these, on average, makes contact with several thousand other neurons. The great majority of synaptic contacts involve a process of chemical transmission in which the arrival of a nerve impulse or an action potential at the terminal region of the axon leads to the influx of Ca^{2+} which in turn mediates the release of a minute amount of chemical transmitter. This chemical messenger rapidly diffuses across the narrow cleft filled with extracellular fluid that separates the nerve terminal from the dendrite or cell body of the neuron with which it communicates. The transmitter then acts upon specialized receptor sites on the surface of the post-synaptic cell to trigger a rapid and short-lasting change in permeability of the cell membrane to certain ions (*e.g.* sodium, chloride). This may lead to depolarization or hyperpolarization of the post-synaptic cell (Iversen and Iversen, 1977; Hille, 1984; Pennartz *et al.*, 1989). Hyperpolarization will stabilize the cell whereas depolarization will lead to the firing of a propagated action potential by the cell due to the existence of ion channels along the axon, which respond to the change in electric potential in their vicinity. In this way, the signal may travel at a speed of up to 100 m/s.

Among other things, the nervous system enables us to respond within a fraction of a second to changes in the environment and continuously informs

us about the position of the body thereby making a coordinated action possible. The latter function is regulated by mechanoreceptors that are located in many tissues and closely connected to the neurons. These receptors are activated upon elastic changes of their membrane resulting in opening of ion channels. The cytoskeleton has been shown to play an important role in sensing the stimulus and in activation of the ion channels (Sachs, 1989).

The functions of the hormone system are very much like the functions of the nervous system. The hormone system coordinates the action of different organs in the body and enables us to respond to the environment. In addition, sex hormones are especially important for survival of the species. The messengers are chemicals (hormones) that are produced by certain tissues and which may be excreted into the blood stream. In this way, the hormones are transported to all the other organs; however, most tissues will not respond due to the lack of hormone receptors (Dufy-Barbe, 1986; Van der Werff ten Bosch, 1985).

The receptor proteins in responsive cells are located at the cell membranes, in the cytoplasm or in the nucleus and generally only 10,000 to 20,000 receptor molecules are present in the cell. A physiological response is the result of a slight change in hormone concentration.

In the case of, for instance, the steroid hormones (*e.g.* oestradiol, progesteron, aldosteron), the chemical diffuses into the cytoplasm and forms a complex with a soluble receptor protein. This results in a change in configuration and thereby activation of the receptor protein. The complex will now bind to the so-called "hormone responsive elements" on the DNA molecules which initiates the transcription of specific RNA-messengers. These in turn may be translated into proteins (enzymes) that mediate the physiological response (Hewitt *et al.*, 1980; Sluyser, 1988). The time elapsed between the secretion of the hormone and the physiological response may vary from minutes up to several hours.

A third way of communication within the body is the immune system. This system especially protects us against pathogenic microorganisms and other alien substances (antigens) by using different classes of leucocytes (white blood cells) and a number of different chemical transmitters (*e.g.* interleukines, interferons).

Upon entering the body, a pathogen is generally first caught by a macrophage (monocyte) that fragments the antigen and incorporates the parts in its

membrane. The macrophage presents the antigen to the T- or B-lymphocytes and may produce interleukine-1, which stimulates the activity of the T- and B-cells and may affect hormone secretion in the hypothalamus.

The B-lymphocytes may react by producing different classes of specific antibodies that become attached to the pathogen. The antigen-antibody complex now attracts different types of complementary factors (proteins) that are activated upon binding. These factors will attack, for instance, the bacterial cell membrane leading to loss of semi-permeability and to death of the microorganism. The T-lymphocytes may react by the production of T-helper or T-suppressor cells which produce chemicals that interact with the production of antibodies by the B-cells (Van den Tweel, 1988; Billian, 1989).

An important feature of the immune system is the formation of T- and B-memory cells. Although at the first infection the production of antibodies may require several days, at a renewed contact with the antigen the memory cells are activated, leading very rapidly to the production of a large amount of specific antibodies. In some cases, a lifetime protection is achieved after the initial infection (Van den Tweel, 1988).

Regulatory mechanisms in plants

Although the metabolism of individual plant and animal cells may show many similarities, there is no *a priori* reason why the mechanism involved in environmental response and in communication between different organs should in any way be the same. Indeed, a nervous system comparable to the animal system has never been found in plants. However, plants like *Drosera* (sundew) or *Mimosa pudica* have a mechanism to respond within seconds to mechanical perturbation. The movements of the leaves of *Mimosa pudica* are regulated by motor organs (pulvini) and are the result of turgor variations in the cortical (motor) cells of these organs. It has been shown that ionic migrations, in particular of K^+ and Cl^-, appear in the pulvinus concomitant with the turgor changes. The cytoskeleton has recently been shown to play a role in the chain of events leading from environmental changes to rapid motor cell response (Satter and Galston, 1981; Fleurat-Lessard *et al.*, 1988).

At present it is generally accepted that plants contain a system that has similarities to the animal hormone system. It involves chemicals of small molecular weight that have a pronounced effect on developmental processes such as growth, flowering, ripening and senescence (Hill, 1980). Up to now, five different classes of these natural growth substances have been iden-

tified and, analogous to animal hormones, these are generally called plant hormones. Of the five classes, two are derived from amino acids, the auxins and ethylene; the others from mevalonic acid: the cytokinins, gibberellins and abscisins (Bruinsma, 1985).

In contrast to animal hormones, which are predominantly produced in specific organs, plant hormones are produced by many individual cells. Therefore, translocation may not be a necessary feature. However, xylem and phloem sap may contain different classes of plant hormones and it is likely that most plant hormones are indeed translocated as messengers within the plant (King, 1976; Bradford and Yang, 1980). In the case of auxins, specific carriers which regulate the movement of auxin in and out of the cell have been identified in the plasma membrane (Libbenga *et al.*, 1986).

The mode of action of plant hormones at the subcellular level is still a matter of debate. Although at present no single receptor protein has been identified with certainty, it is generally believed that the mechanism shows many similarities to the situation in animals. The hormone is thought to bind to a receptor protein, after which the hormone-receptor complex or a second messenger interferes with the transcription process, leading to changes in the production of messenger RNA's (Libbenga *et al.*, 1986). Unlike animal hormones, it is most likely that a physiological response to a plant hormone is achieved not exclusively from a change in hormone concentration but also from a change in sensitivity of the responding tissue (Trewavas, 1981; Starling *et al.*, 1984). The sensitivity may depend on, *e.g.*, the amount or accessibility of receptor proteins.

Apart from the five classes of plant hormones, other substances may probably be added to the list as a number of developmental processes cannot be fully explained with the presently known substances.

Upon infection with microbes, most plants show an accumulation of antimicrobial compounds of low molecular weight at the site of infection. At present, more than a hundred of such plant-protecting substances, called phytoalexins, have been isolated from a large number of plant species.
These compounds (*e.g.* sesquiterpenoids, isoflavonoids) are rapidly produced by the host cells and it has been established that in many cases *de novo* synthesis of RNA-messengers and enzymes is apparent prior to the increase in phytoalexin concentration.

Phytoalexins mostly originate from shikimate, acetate or mevalonate and a single plant may require as many as 20 enzymes to synthesize them. Most of

these enzymes are absent in healthy, non-infected plants. The accumulation of phytoalexins may, for instance, inhibit fungal growth or kill the host or microbial cells thereby isolating the infection in favor of the rest of the plant (Darvill and Albersheim, 1984; Érsek and Király, 1986).

The molecules that signal the plant to start the process of phytoalexin synthesis are called elicitors. Elicitors may be constituents of microbes (*e.g.* complex hydrocarbons) or may be released from the plant cell wall by enzymes secreted by the infecting microbes or by the plant cell itself. Furthermore, abiotic factors such as heavy metals or UV-light may also elicit phytoalexin production. In what way the elicitors turn on the expression of selected genes and how they may inhibit the expression of other genes is at present unknown (Davis and Hahlbrock, 1987; Baldwin and Pressey, 1988).

In different systems it has been established that one of the responses to infection is an increased production of ethylene that, in turn, may further stimulate the phytoalexin production. Although ethylene itself may serve as an elicitor, it is most probably not a prerequisite for phytoalexin production (Paradies *et al.*, 1980; Ecker and Davis, 1987).

In interactions between host plants and microbes or viruses an induced systemic resistance in non-inoculated plant parts may be found following the initial infection (Van Loon, 1976). This has been associated with the synthesis of "pathogenesis-related" proteins (for instance chitinases, 1,3-β glucanases) in the non-infected areas. Since chitin and 1,3-β glucan are the main cell wall components of many potentially pathogenic fungi such a systemic resistance would protect the plant against further infection. As in the case of enzymes involved in the production of defense-related phytoalexins, the regulation of pathogenesis-related proteins is probably at the transcriptional level (Vögeli *et al.*, 1988; Joosten and De Wit, 1989; Lawton and Lamb, 1987).

Ethylene biosynthesis and action

The unsaturated gaseous hydrocarbon ethylene (C_2H_4) plays an important role in many developmental processes. In growing organs it generally inhibits growth while, in mature organs, ripening, senescence and abscission is promoted. In addition, ethylene may stimulate seed germination and the formation of female flowers in some plant families (Abeles, 1987).

Ethylene is synthesized from methionine through the intermediates S-adenosyl methionine (SAM) and the cyclic amino acid 1-aminocyclopropane-1-carboxylic acid (ACC). The conversion of SAM to ACC and methylthioadenosine

(the latter being recycled into methionine) is generally believed to be the rate-limiting step in ethylene biosynthesis (Yang and Hoffman, 1984). The enzyme involved, ACC-synthase, is located in the cytoplasm, requires pyridoxal phosphate and is readily induced by all sorts of stress conditions including exposure to ethylene. It has a half-life of about 20 to 30 min thereby providing a mechanism for rapid regulation of ethylene biosynthesis.

The conversion of ACC to ethylene, HCN and CO_2 is catalysed by a membrane-bound enzyme system known as the ethylene-forming enzyme (EFE). This oxidative enzyme requires free radicals and a more or less intact cell (Yip *et al.*, 1988; John *et al.*, 1989). The formed HCN is subsequently detoxified by incorporation into aspartic acid. EFE-activity is thought to be largely constitutive in most plant tissues and may be stimulated by carbon dioxide and ethylene. A half-life of several days has been reported (Kende *et al.*, 1985, Manning, 1986).

In most systems, ACC is simultaneously converted into N-malonyl ACC (MACC). The enzyme involved is not a specific one but converts all D-amino acids into their malonyl derivatives. The lack of stereo-chemistry of ACC is the reason that it is also "detoxified". The malonyl transferase was shown to be regulated by ethylene and by light. MACC is believed to be an inactive end product predominantly transported into the vacuole. It is thought not to be converted into ACC under natural conditions (Amrhein *et al.*, 1982; Van Loon and Fontaine, 1984; Pech *et al.*, 1989).

An interesting feature of the ethylene-biosynthetic pathway is that the enzymes involved, particularly ACC-synthase, may be stimulated by ethylene, leading to a sudden upsurge in ethylene production known as autocatalysis (Yang and Hoffman, 1984). The enhanced level of endogenous ethylene may surpass threshold values for the induction of such phenomena as climacteric respiration and enzyme production in, *e.g.*, ripening fruits. However, in some cases, autoinhibition through suppression of ACC-synthase activity or stimulation of malonyl-transferase activity may also occur (Riov and Yang, 1982; Philosoph-Hadas *et al.*, 1985).

Once produced, ethylene is thought to bind, presumably in a reversible way, to a receptor protein. Binding results, via a yet unknown chain of events, in the transcription of specific RNA-messengers and the production of enzymes that are responsible for the physiological effect (Woodson and Lawton, 1988). Plant cells may contain several tens of thousands of ethylene binding sites and the number may vary during development. However, it has never been shown that the responsiveness of certain tissues is dependent on

the actual number of binding sites present (Sisler and Goren, 1981; Hall, 1986; Brown *et al.*, 1986).

Oxidation of ethylene into ethylene-oxide, ethyleneglycol and carbon dioxide has also been reported and it has been suggested that ethylene action may require ethylene oxidation. However, the following observations do not support this view (Raskin and Beyer, 1989):

1) the enzyme involved, ethylene mono-oxygenase, is also present in systems that do not show a physiological response to ethylene (bacteria, humans);
2) inhibition of the enzyme by CS_2 or cyclic olefins does not inhibit ethylene action.

Alternatively, it has been suggested that ethylene oxidation may serve as a detoxification mechanism for ethylene. This view, however, may also be wrong as treatments that completely block the enzyme do not enhance the rate of ethylene evolution (Raskin and Beyer, 1989).

Senescence of flowers

Nichols (1966) was the first to show that wilting in carnation flowers was accompanied by a huge upsurge in ethylene production. Thereafter, it was confirmed that many other flower species show similar increases in ethylene production during senescence. In such species, treatment with low concentrations of ethylene generally hastens senescence and the appearance of the accompanying upsurge in ethylene production.

Sensitivity to exogenous ethylene is generally determined at the family level (Woltering and Van Doorn, 1988). In a sensitive species ethylene advances the appearance of the natural senescence symptoms and, in addition, may cause flower malformations or stimulate microbial attack (Woltering, 1987; Woltering and Van Doorn, 1988). The responsiveness to ethylene depends, among other factors, upon carbohydrate status, developmental stage, temperature, exposure time and the concentrations of ethylene, carbon dioxide and oxygen (Mayak and Dilley, 1976; Camprubi and Nichols, 1978; Woltering and Harkema, 1987; Smith and Parker, 1966; Burg and Burg, 1967).

In ethylene-sensitive species, pollination and several types of stress (*e.g.* water loss, dark storage) were shown to hasten senescence. It may therefore be argued that such an effect is due to a stress-induced increase in ethylene production and/or ethylene sensitivity. For instance, water loss in harvested carnation flowers advances the onset of the increase in ethylene production accompanying senescence. During water stress and following the release from stress no changes in ethylene production were detected, but

senescence-associated changes in the cell membranes were shown to occur shortly after application of the stress (Coker *et al.*, 1985; Paulin *et al.*, 1985). These and other observations led to the conclusion that stress influences the permeability of the cell membranes which in turn leads to accelerated ethylene production and senescence. Dark storage, as another example, is known to induce abscission of flowers and flower buds in several species. In the case of *Lilium*, dark-induced bud abscission was accompanied by an increased ethylene production that was autocatalytic in nature, indicating that ethylene sensitivity increased in the dark (Van Meeteren and De Proft, 1982).

In particular the dramatic effect of pollination and stigma wounding on longevity and ethylene production has been intensively studied. In *Petunia*, for instance, there is an increase in ethylene production almost immediately after the pollen grains have touched the stigma. Although pollen may contain considerable amounts of ACC, the early ethylene production in these flowers is a result of an increased ACC-synthase activity in the stigma (Hoekstra and Weges, 1986; Pech *et al.*, 1987). The early peak in ethylene production is followed some days later by a second increase, concomitant with wilting of the corolla. The early pollination or wound-induced ethylene production was found to be exclusively produced by the gynoecium; the second peak by the corolla (Hoekstra and Weges, 1986).

When the style and stigma were removed within 6 to 8 h after pollination, the wilting of the corolla was considerably hastened in comparison to non-pollinated flowers. This indicates that already during the first hours after pollination or wounding a substance is transported over a distance of several centimeters from the stigma to the corolla which leads to an earlier appearance of the increase in corolla ethylene production and senescence of the flower (Gilissen and Hoekstra, 1984). Although direct proof was lacking, Hoekstra and Weges (1986) argued that ACC was most probably the transported wilting factor in this species. However, no ACC could be detected in eluates from pollinated styles (Gilissen and Hoekstra, 1984).

It has also been suggested that pollination may lead to the production of a mobile substance that may move from the stigma to the corolla, making the latter more sensitive to ethylene. Only very recently, two possible candidates, *i.e.* the short-chain saturated fatty acids decanoic acid and octanoic acid, were isolated from pollinated styles and it was shown that these fatty acids may indeed be rapidly translocated within the flower (Whitehead and Halevy, 1989). Such a pollination-induced ethylene-sensitivity factor was also suggested to be involved in flower abscission in *Cyclamen* (Halevy *et*

al., 1984). Furthermore, in carnation petals the basal part is thought to produce an ethylene-sensitivity factor that is transported to the upper part of the petal (Mor *et al.*, 1985). Similar changes in ethylene sensitivity have also been suggested to occur during ripening of certain fruits (McGlasson, 1985; Knee, 1988).

Outline of the thesis

During post-harvest life, most horticultural products are continuously exposed to all sorts of stress that may shorten their shelf life. The mechanism of stress-induced senescence, however, is still poorly understood. Initially it may involve a change in ethylene-biosynthetic activity or ethylene sensitivity while specific mobile wilting or ethylene-sensitivity factors may also play a role. Alternatively, ethylene may only play a role during the later stages of senescence.

Emasculation (removal of the pollinia and the anther cap) of orchid flowers has been shown to hasten the senescence process, leading to premature wilting and, in *Cymbidium*, also to red coloration of the labellum (Burg and Dijkman, 1967; Arditti *et al.*, 1973). In nature, emasculation is carried out by insects, while searching for nectar in the flower, or by mice that feed on these flower parts. During postharvest life, emasculation may occur as a result of handling. For a better understanding of the events responsible for stress-induced senescence, the effect of emasculation on senescence-associated biochemical changes in orchid flowers was studied.

This thesis discusses the use of a new system for the determination of low concentrations of ethylene and provides kinetic data concerning the early changes in ethylene production during senescence of flowers (chapters 2 and 3). The use of the emasculated *Cymbidium* flower as an experimental model to study stress-induced senescence and the regulation of anthocyanin synthesis in the labellum is described in chapters 4 and 5. Chapter 6 presents evidence that desiccation of the rostellum is the primary event in emasculation-induced senescence. In chapters 7 and 8 the regulation of senescence and the roles of ethylene and ACC in inter-organ communication are discussed. Chapter 9 describes the effect of column eluates on coloration of isolated lips. Chapter 10 gives a general discussion.

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CHAPTER 2

USE OF A LASER-DRIVEN PHOTOACOUSTIC DETECTION SYSTEM FOR MEASUREMENT OF ETHYLENE PRODUCTION IN *CYMBIDIUM* FLOWERS

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SUMMARY

A laser-based photoacoustic method was used for determination of ethylene (C_2H_4) production of emasculated orchid *(Cymbidium)* flowers in a flow-through system. The laser photoacoustic equipment consisted of a line-tuneable CO_2-laser in conjunction with a single-pass resonant acoustic cell. The minimum detection limit of the system for ethylene in air was 0.03 nl/l.

The ethylene production of intact *Cymbidium* (cv. Mary Pinchess 'Del Rey') flowers was very low (0.015 nl/g.h) but showed an increase within 3 h following emasculation (removal of pollinia plus anther cap). Production peaked (0.14 nl/g.h) 8 h after emasculation and decreased thereafter. Production again increased 45 h after emasculation. Coloration of the labellum appeared shortly after the first peak; wilting of the petals and sepals appeared during the second rise in ethylene production. The use of the laser photoacoustic technique in plant physiological studies is discussed.

Plant Physiology 88:506-510(1988).

Since Neljubov (1901) discovered that ethylene was the physiologically active component of illumination gas, many plant physiologists have become interested in this gaseous plant growth regulator. Gane (1934), using chemical methods for analysis, showed that plants themselves produced ethylene. A sensitive manometric method for determination of ethylene was introduced by Young *et al.* (1952) and a few years later Burg and Stolwijk (1959) introduced a gas chromatographic technique. Nowadays, analysis of ethylene by means of a gas chromatograph (GC) equipped with a flame ionization detector (FID) has become a standard procedure in most laboratories. The typical minimum detection limit of such an apparatus is 5-10 nl/l (signal:noise = 2:1).

For measurement of ethylene production in intact shoots and ornamental products such as flowers, a standard GC is suitable when the products are placed for a prolonged period of time in a closed environment, thereby allowing produced ethylene to accumulate. A sample of the head space is then analysed for ethylene. This approach has a number of intrinsic disadvantages. The rate of ethylene production may be influenced by the changing concentrations of carbon dioxide and oxygen, while either autocatalysis or autoinhibition might also interfere (Dhawan *et al*, 1981; Kao and Yang, 1982; Bassi and Spencer, 1983; Yang and Hoffman, 1984). Furthermore, the accumulation will mask rapid changes in ethylene production that might be of physiological importance.

These problems can be overcome through the use of a GC equipped with a photo ionization detector (PID) and, to a lesser extent, through the use of a system capable of controlling the carbon dioxide and oxygen concentrations (Woltering and Sterling, 1986). The PID's are several-fold more sensitive than FID's and have even been used to measure ethylene production of plants in a flow-through system. (Bassi and Spencer 1985). In another approach, collection of the gas is achieved by trapping it on a solid support maintained at a low temperature, as described by De Greef *et al.* (1976) and by Bassi and Spencer (1979). Although long sampling intervals are necessary in some cases, these methods allow measurements of ethylene production of intact plants in a flow-through system.

In the last decades, laser photoacoustic (LPA) spectroscopy has been used for detection of atmospheric pollutants. High power, step-tuneable CO and CO_2 lasers have been frequently used and, in principle, more than 250 gases of environmental interest (including ethylene) could be investigated with such a

laser (Hubert, 1983; Perlmutter *et al.*, 1979).

The photoacoustic effect was discovered by A.G. Bell (1881) and has gained renewed attention following the advent of powerful infrared lasers. In photoacoustics, for instance, the radiation source (*e.g.* a CO_2 laser beam) is periodically interrupted by means of a mechanical chopper. The chopped beam is directed into a small vessel (PA cell) containing for example a gaseous sample assumed to absorb at the emission frequencies of the laser.

Following the absorption of energy the gas molecules are excited from the ground state into a rotational level in a higher vibrational state and de-excitation processes will then redistribute the energy. In the infrared region the probability for radiative decay is small and, at atmospheric pressure, relaxation generally takes place along the non-radiative channel. This causes an increase in kinetic energy of gas molecules and, hence, also of gas temperature. Consequently, in a closed (or semi-closed) vessel of constant volume, the increase in temperature leads to a corresponding increase in pressure. When the radiation source providing the energy is modulated at audio frequency, the generated pressure changes (acoustic waves) can be detected by a microphone.

We report here on an LPA system for measurement of ethylene production of ornamental products in a flow-through system. Orchid (*Cymbidium*) flowers were chosen because intact flowers show a low ethylene production that can be manipulated by removal of the pollinia (emasculation), which includes removal of the anther cap (Goh *et al.*, 1985).

MATERIALS AND METHODS

LPA detection system

The LPA detection system used in this study consists of a modified version of a line-tuneable infrared CO_2 laser (Strumia and Ioli, 1985) and a stainless steel "organ pipe" single-pass resonant PA cell (Scherer *et al.*, 1984). Most of the equipment was constructed in the laboratories of the Catholic University Nijmegen.

The used CO_2 waveguide-laser operates in a direct current longitudinal discharge regime on a flowing mixture of He, N_2 and CO_2 (ratio 6:1:1) as active medium. In the laser tube the typical operating pressure is 80 mbar. The maximum output power of the laser amounts to 6 W at the used frequencies and more than 80 discrete laser wavelengths between 9 and 11 μm can be selected

(Harren *et al.*, 1987). Selection of the desired wavelength was achieved by adjusting the position of the diffraction grating by means of a micrometer driven by a stepmotor. The laser beam was periodically interrupted (mechanically) by a phase-stabilized chopper.

In order to get a maximum signal, the chopper was tuned to the resonance frequency of the PA cell filled with a mixture of ethylene in air (572 Hz). At this frequency a "standing-wave" is generated in the inner, open resonator of the PA cell (Fig. 1; length = 300 mm, diameter = 9 mm). The resonator in turn is mounted with airtight teflon spacers in a larger tube. In this way the laser-induced heating of the cell windows does not couple efficiently into the main resonator, thereby minimizing its effect on the PA signal. The acoustic signal is detected by four miniature microphones (Knowless, type BT 1754) mounted in 1 mm diameter holes in the middle of the resonator. The microphone signal is further processed by synchronous detection at modulation frequency using a lock-in amplifier (EG & G, Princeton, NJ, USA).

The responsivity of the PA cell was determined using a 95 nl/l certified mixture of ethylene in N_2 (Matheson). In other experiments (data not shown), it was shown that the PA signal was strictly linear with the concentration of ethylene (0.05 up to 1000 nl/l).

In an actual experiment, the relevant signals were fed to an Apple IIc computer for calculation of the ethylene concentration.

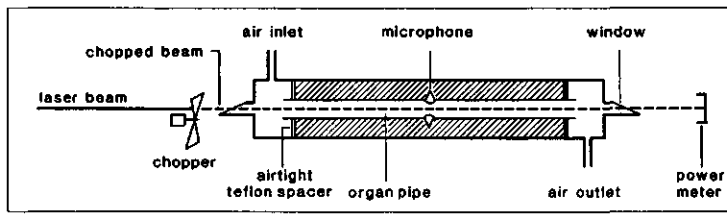

Fig. 1. Schematic presentation of the laser photoacoustic detection system.

Measurement of ethylene production

Ethylene production of individual *Cymbidium* flowers was measured in a flow-through system (flow rate 0.9 l/h) consisting of small glass cuvettes (volume 80 mL) fitted with in and outlet ports (Fig. 2). The cuvettes were also provided with a septum-stoppered port to facilitate the emasculation treatment. A copper-constantan thermocouple connected to a data-logger was inserted in each cuvette to monitor temperature. Relative humidity (RH)

inside the cuvettes was regularly measured using a "Murata" dew sensor coupled to an ohm-meter. The experiments were carried out under continuous light of about 15 μmol/m^2.s.

Compressed air (0.035% CO_2; v/v) was first passed through a copper tube (6 m) filled with a platinized aluminium oxide catalyst (at 350°C) to remove hydrocarbons. Through a flow-controller (Brooks) the purified air was admitted into the cuvettes.

Air from the cuvettes passed through a mass-flow meter (Brooks) and an infrared carbon dioxide monitor (ADC) both connected to a data-logger, in order to monitor flow and CO_2 concentration, respectively. The air was then passed through a glass column (10 x 1 cm) filled with KOH grains to remove water and CO_2. Thereafter, the air was passed through the PA cell. When not in line with the PA cell, the effluent was vented to atmosphere.

The photoacoustic signal was measured at two laser wavelengths (10.51 and 10.53 μm) at which the absorption coefficients of ethylene are known (Brewer *et al.*, 1982).

The experiments were repeated several times with essentially identical results.

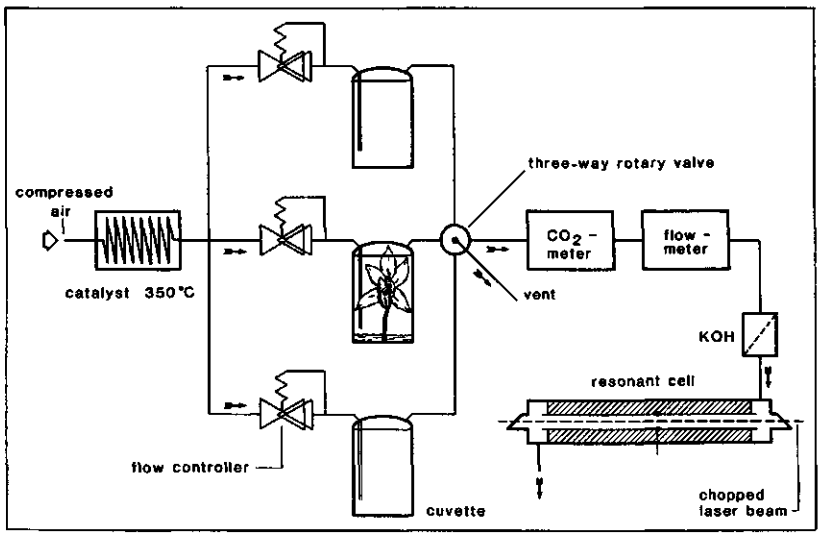

Fig. 2. Schematic presentation of the experimental setup for measurement of ethylene production.

Plant material

Cymbidium flowers (cv. Mary Pinchess 'Del Rey'), obtained from commercial growers, were placed in the cuvettes well before start of the measurements in order to acclimatize. Each cuvette contained one flower placed in ca. 10 ml water to avoid desiccation. By means of a lever (paperclip), fitted in a silicon rubber septum, emasculation (removal of pollinia plus anther cap) was carried out from the outside without changing the gaseous composition inside the cuvette.

RESULTS

The typical CO_2 laser photoacoustic spectrum of ethylene is shown in figure 3. Very high absorption (high microphone signal) was found at 10.53 μm while absorption at the adjacent laser transition (10.51 μm) was much lower. The ethylene concentration was calculated from the microphone signal recorded at both discrete laser transitions, thereby eliminating possible interpretation errors due to the effect of interfering volatiles (such as odors) that generally have comparable absorption over a broad range of laser transitions.

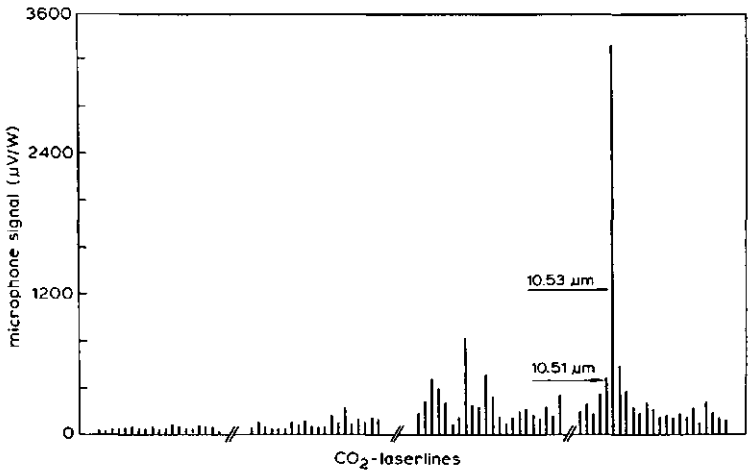

Fig. 3. Photoacoustic signal at 84 discrete laser transitions between 9 and 11 μm of a gas mixture containing 1000 nl/l ethylene in N_2.

The flow characteristic of the system was determined by injecting a known amount of ethylene into an empty cuvette that was flushed with purified air at a flow rate of 0.9 l/h. The PA signal was monitored during a period of 1 h after injection (Fig. 4). The half response time was found to be about 10 min; the signal reached its initial level after approximately 1 h. The total volume of the system (including the PA cell) was calculated to be 225 ml. At this flow rate, switching between the various cuvettes will only yield valuable information if a sampling interval of at least 45 min is sustained.

When a *Cymbidium* flower was placed in the cuvette and an amount of water was added, the outlet air contained about 0.045% CO_2 (v/v) and was nearly saturated with water vapor (about 90% RH). The glass column packed with KOH grains reduced the CO_2 concentration and water vapor pressure to about 1 μl/l and 1 Torr, respectively. At these low levels, the contribution of these constituents to the PA signal is negligible. It was checked that the KOH grains did not retain any ethylene.

During the experiments no significant fluctuations in the air flow were found (less than 5%), the temperature fluctuations were within 1.0°C.

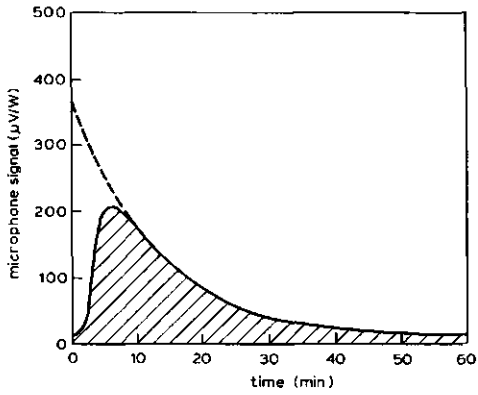

Fig. 4. Characteristic of the flow-through system (flow rate = 0.9 l/h). At t = 0, ethylene was injected into the cuvette.

Figure 5 represents data from two cuvettes containing *Cymbidium* flowers. The rotary valve (see Fig. 2) was operated alternatively between a cuvette with an intact flower and a cuvette with an emasculated flower. Each cuvette was connected for a period of 45 min to the PA cell.

Changing from one cuvette to the other produces a rapid change in ethylene concentration until, after about 40 to 45 min, the actual concentration in the cuvette is reached. This experiment shows that sampling intervals of about 45 min are indeed necessary to obtain reliable measurements of the ethylene evolution. The ethylene concentration (expressed as the microphone signal) in the outlet of the emasculated flower showed an increase and a decrease during this experiment; the ethylene concentration from the intact flower showed no significant fluctuations.

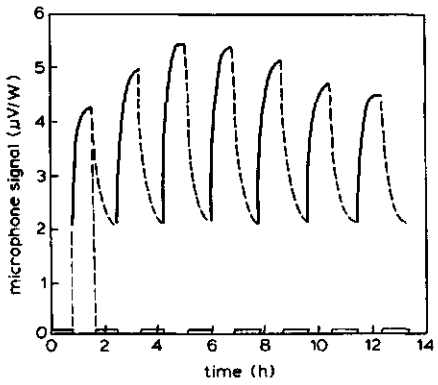

Fig. 5. Characteristic of the flow-through system (flow rate = 0.9 l/h). Ethylene concentrations (expressed as microphone signal) from a cuvette with an intact *Cymbidium* flower (---) and from a cuvette with an emasculated *Cymbidium* flower (——) were measured for periods of 45 min. Emasculation was carried out 2 h before the start of the measurements.

In another experiment, the ethylene concentration at the outlet of a cuvette with an emasculated flower and of an empty cuvette was measured. The ethylene concentration at the outlet of the empty cuvette was approximately 0.4 nl/l and showed no significant changes during the course of the experiment (data not shown). The difference in measured ethylene concentration between the two cuvettes is the amount of ethylene produced by the flower (Fig. 6; left ordinate). From these data, ethylene production (nl/g FW.h) was calculated (Fig. 6; right ordinate).

The calculated ethylene production was found to be very low before emasculation (0.015 nl/g.h) with a relatively minor, but readily detectable change within 3 after emasculation. The production peaked (0.14 nl/g.h) at about 8 h after emasculation and showed a gradual decline thereafter. The production rose again about 45 h after emasculation.

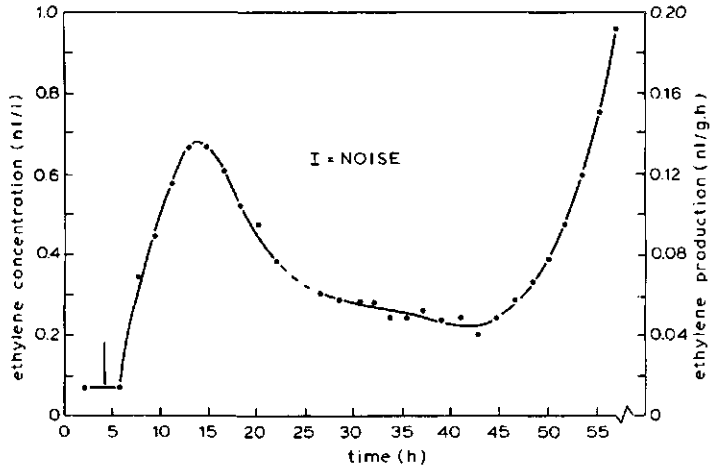

Fig. 6. Calculated ethylene production (right ordinate) and difference between ethylene concentration in the outlet air from an empty cuvette and from a cuvette with a single *Cymbidium* flower (left ordinate) as a function of time. Arrow indicates time of emasculation. After 24 h the experiment was interrupted briefly to change the laser supply gases.
Flow rate = 0.9 l/h; temperature = 22°C.

DISCUSSION

During the last decade, PA detection of photosynthetic activities has become a valuable tool in plant science. Applied to a leaf, the method yields information about the conversion of light into heat and, hence, indirectly about the conversion of light into chemical energy. Furthermore, it has been found that PA signals also reflect oxygen evolution in the leaves (*e.g.* Buschmann *et al.*, 1984; Canaani and Malkin, 1984; Canaani, 1986). To our knowledge, no reports are available about the use of PA systems for detection of ethylene emanation (from plants) in plant physiological studies.

The laser-driven PA system used in our study was extremely sensitive for determination of ethylene. The minimum detection limit was 0.03 nl/l in the present experiments, which is much more sensitive than any other method described. While concentrations were well below the nl/l level, on-line detection of ethylene directly in the outlet of a flow-through system (flow rate = 0.9 l/h) with a single *Cymbidium* flower appeared possible, without accompanying problems arising from an inadequate control of the gaseous composition in the cuvette.

The relatively large volume of the PA cell, however, was a disadvantage in our experiments since, due to the flow characteristic of the system, interchanging between various cuvettes was possible only once per 45 min. Constructing a smaller-sized PA cell is theoretically possible and will eliminate this problem. In addition, it is also still possible to considerably improve the sensitivity of the LPA system either by using more sensitive microphones and a more powerful laser or by placing the PA cell inside the laser cavity. With such a system, a sensitive detection at sampling intervals of several minutes becomes accessible.

An important advantage of the LPA method in comparison to the use of a GC either with PID or FID and collection unit is the direct character of detection, which makes it possible to accurately measure small changes in concentration upon a background of several nl/l ethylene. The use of extremely pure air (< 0.1 nl/l ethylene), which is a prerequisite for proper functioning of the trapping methods, also introduces a steep concentration gradient between the site of ethylene synthesis and the gaseous phase that might alter the pattern of ethylene synthesis within the tissue (Smith and Hall, 1984).

In a number of flowers, senescence is thought to be mediated by endogenous ethylene production. Flowers like carnations (Nichols, 1966) and various orchid species (Burg and Dijkman, 1967; Goh *et al.*, 1985) show a large increase in ethylene production prior to or during the wilting process. It may be suggested, however, that this increase in ethylene production although necessary for integration of the wilting process is not the initial event in senescence because changes in membrane permeability and microviscosity have been observed well before the onset of the ethylene peak (Coker *et al.*, 1985; Eze *et al.*, 1986).

Since treatment with exogenous ethylene generally hastens both the onset of the increase in ethylene production and the changes in membrane properties, we suggest that minor fluctuations in the basal ethylene production might play a role in triggering the senescence processes. The validity of this hypothesis can only be tested with very sensitive methods.

The presented results on ethylene production in emasculated *Cymbidium* flowers demonstrate two distinct, readily detectable peaks following emasculation. The first peak is relatively small and has apparently not been detected by others who studied the ethylene production in emasculated *Cymbidium* flowers (Chadwick *et al.*, 1980; Goh *et al.*, 1985). The first peak coincides with coloration of the labellum, a phenomenon which is generally observed within one day after emasculation (Chapter 4). The second increase in

ethylene production coincides with wilting of the sepals and petals. In intact flowers these symptoms (coloration and wilting) manifest themselves after a much longer time span.

It is therefore suggested that the first (relatively small) peak in ethylene production following emasculation initiates both the synthesis of anthocyanins in the labellum and the autocatalytic process responsible for wilting of the sepals and petals in these flowers. This view is supported by the observation that treatment of these flowers immediately after emasculation with the gaseous inhibitor of ethylene action, 2,5-norbornadiene (Sisler and Yang, 1984) at 1000 μl/l considerably delays the development of emasculation-induced coloration and wilting (data not shown).

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CHAPTER 3

LASER PHOTOACOUSTIC DETECTION OF INITIAL CHANGES IN ETHYLENE PRODUCTION DURING SENESCENCE OF DIFFERENT CUT FLOWER SPECIES

Ernst J. Woltering, Frans Harren and Dane D. Bicanic

SUMMARY

A CO_2 laser-based photoacoustic method was used to determine the early changes in ethylene production during senescence of cut carnation (*Dianthus caryophyllus*) and orchid (*Phalaenopsis*, *Cymbidium*, *Oncidium splendidum*, *Epidendrum*) flowers. In all tested species, a small change in the basal ethylene production was detectable well before the start of the autocatalytic upsurge that accompanies senescence of the flowers. In *Cymbidium* coloration of the lip and an increased leakage of chloride ions from petal tissue pre-loaded with radioactive hydrogenchloride appeared following the emasculation-induced early peak in ethylene production. It is therefore suggested that minor changes in ethylene production may initiate senescence processes including an increase in membrane permeability and autocatalytic ethylene production.

The data discussed in this chapter have been published in two separate papers.

1. E.J. Woltering, F. Harren and D.D. Bicanic. Laser photoacoustics: A novel method for ethylene determination in plant physiological studies. **Acta Horticulturae 261:201-208(1989).**
2. E.J. Woltering and F. Harren. Early changes in ethylene production during senescence of carnation and *Phalaenopsis* flowers measured by laser photoacoustic detection. In: Clysters *et al.* (eds.), Biochemical and Physiological Aspects of Ethylene Production in Lower and Higher Plants. **Kluwer Academic Publishers, 1989, pp. 263-270.**

INTRODUCTION

Senescence in a number of different cut flowers (*e.g.* roses, carnation, *Petunia*, various orchids) is accompanied by a sudden increase in their endogenous ethylene production (Nichols, 1966; Whitehead *et al.*, 1984; Faragher and Mayak, 1984; Goh *et al.*, 1985). This increase, which is also observed during ripening of climacteric fruits, is thought to be autocatalytic in nature and the result of an ethylene-stimulated increase of the activity of enzymes involved in ethylene biosynthesis (Yang and Hoffman, 1984). Although presumably necessary for integration of the ripening process(es), this autocatalytic upsurge observed in fruits may not be the primary event. Changes in ethylene sensitivity rather than ethylene production may initiate the autocatalysis (McGlasson, 1985; Knee, 1988).

In cut roses it has been demonstrated that endogenous ethylene plays an important role in the initiation of senescence (Faragher and Mayak, 1984). Following the rise in ethylene production, an increased membrane permeability in petal tissue of these flowers is observed. From inhibitor studies it has been concluded that the low, basal ethylene production may already have an effect on membrane integrity (Faragher *et al.*, 1987). In carnation petals, however, changes in membrane properties were observed well before the onset of the autocatalytic ethylene production. Ethylene may therefore be the result of senescence processes that are initiated by a yet unknown factor, rather than the trigger (Trippi and Paulin, 1984; Eze *et al.*, 1986; Sylvestre and Paulin, 1987).

In carnation, several types of stress *e.g.* exogenous ethylene or water stress are known to advance the appearance of the sudden rise in ethylene production and the accompanying senescence symptoms (Mayak *et al.*, 1977; Borochov *et al.*, 1982; Coker *et al.*, 1985; Paulin *et al.*, 1985). In orchids the ethylene peak is advanced by emasculation, *i.e.* removal of the pollinia including the anther cap (Burg and Dijkman, 1967; Goh *et al.*, 1985).

To test the hypothesis that stress-induced changes in ethylene production may initiate the senescence processes, including the autocatalytic upsurge, we used a very sensitive laser-driven photoacoustic (LPA) system for the measurement of ethylene (see appendix).

MATERIALS AND METHODS

Plant material

Flowers of carnation *(Dianthus caryophyllus)* cv. Bagatel, *Phalaenopsis* (hybrid) cv. Red Lip and *Cymbidium* cv. King Arthur were obtained from commercial growers and transported dry to the laboratory; the other orchid species (*Oncidium splendidum* and *Epidendrum* (hybrid)) were collected in the glasshouses of the Catholic University Nijmegen and immediately used for the experiments.

Ethylene production

Ethylene evolution of individual, mature flowers was measured under continuous flow conditions using a laser photoacoustic detector. The laser photoacoustic equipment consisted of a line tuneable CO_2 laser in conjunction with a single-pass resonant acoustic cell. The minimum detection limit for ethylene was 0.03 nl/l.

Flowers with a stem length of ca. 2 cm, standing in 3 ml vases, were placed in glass cuvettes (80 ml) and were flushed with purified air (< 0.5 nl/l ethylene) at a flow rate of 0.9 l/h which prevented the accumulation of carbon dioxide and ethylene. A detailed description of the experimental set-up has been described by Woltering *et al.* (1988).

Determination of ion-leakage

Individual flowers (*Cymbidium* cv. King Arthur) were placed with their stems in a solution of 2.5×10^5 Bq $H^{36}Cl$. After ca. 24 h the flowers were transferred to water after which some were emasculated. One and two days after emasculation small discs, excised from the petals, were first immersed in water for ca. 15 min to wash away the solutes from the wounded cells and thereafter incubated in 5 ml water at 35°C and continuously shaken.

At intervals, 100 µl samples were taken from the incubation solutions for determination of radioactivity by liquid scintillation counting. After ca. 6 h of incubation, the tissue was exposed to two cycles of freezing and thawing after which the total amount of radioactivity released from the discs was determined. Leakage of $^{36}Cl^-$ was expressed in % of the total amount of radioactivity in the discs.

All experiments were repeated at least once and representative data are shown.

RESULTS

The ethylene production of carnation flowers showed a number of clearly discernible phases (Fig. 1).

Phase I: A low constant level of about 0.05 nl/g.h during the first four days of vase life.

Phase II: A gradual increase from 0.05 to 0.1 nl/g.h during the fifth day.

Phase III and phase IV: A much faster increase.

Regression analysis showed that phase II was well described by a linear relation between ethylene production and time; phase III was well described by a log-linear relation (Fig. 1). Plotting of the data from this experiment on a semi-logarithmic scale showed that indeed, phase II and phase III may not be regarded as part of one and the same log-linear relation (Insert Fig. 1). Following phase III, the production again showed a linear relation with time (phase IV; not shown). After the production reached its top (~ 18 nl/g.h), a gradual decrease was seen during the next few days to a low level (data not shown).

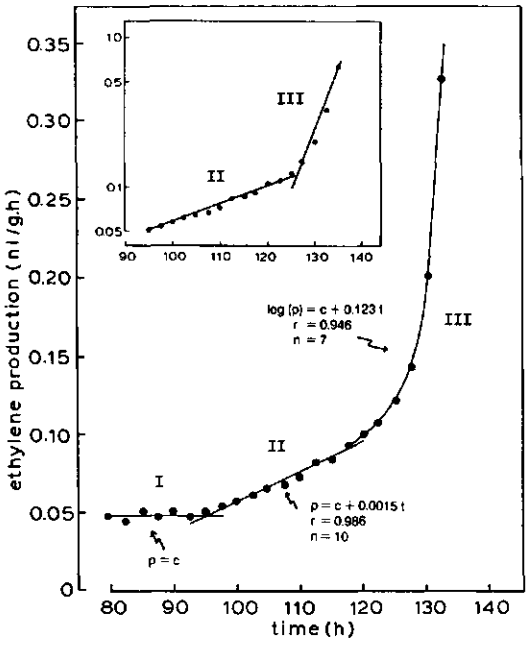

Fig. 1. Ethylene production (p) of carnation cv. Bagatel plotted as a function of time after cutting (t). c = constant; r = correlation coefficient; n = number of observations.
Insert: Ethylene production plotted on a logarithmic scale.

The ethylene production of a (non-emasculated) *Cymbidium* flower is shown in figure 2. The production was initially very low (0.025 nl/g.h; phase I). A gradual linear increase (phase II) appeared during the 6th and the 7th day which coincided with coloration of the lip. A much more pronounced log-linear increase (phase III) appeared during the 8th day and thereafter a linear increase was again observed (phase IV). The semi-logarithmic presentation of these data clearly shows the different phases (Insert Fig. 2).

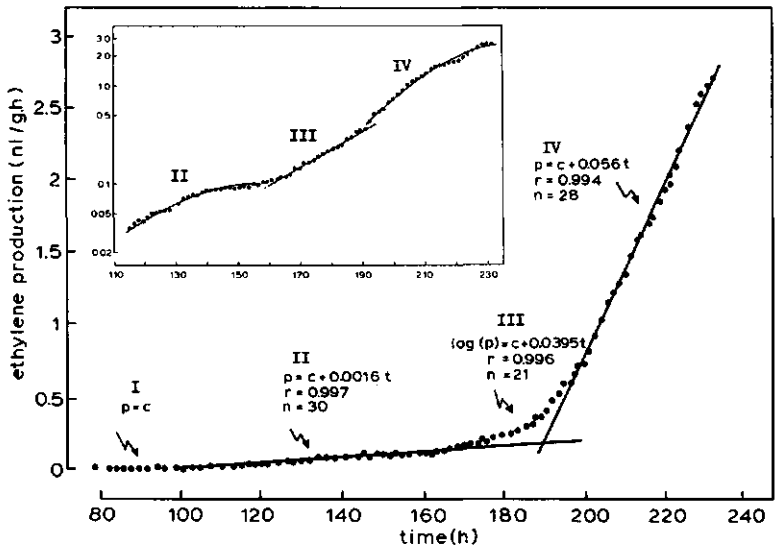

Fig. 2. Ethylene production (p) of *Cymbidium* cv. King Arthur plotted as a function of time after cutting (t).
Insert: Ethylene production plotted on a logarithmic scale.

In *Phalaenopsis* flowers the ethylene production was very low (about 0.015 nl/g.h) prior to emasculation (Fig. 3), approximately the detection limit of the system in the present experiment. Within a few hours after emasculation the production showed a gradual (linear) increase from 0.015 to 0.2 nl/g.h (phase II) which developed into a much faster (log-linear) increase (phase III). Following the log-linear phase, the production again showed a linear increase until it reached its top (~ 15 nl/g.h). Thereafter a gradual decrease was observed (data not shown).

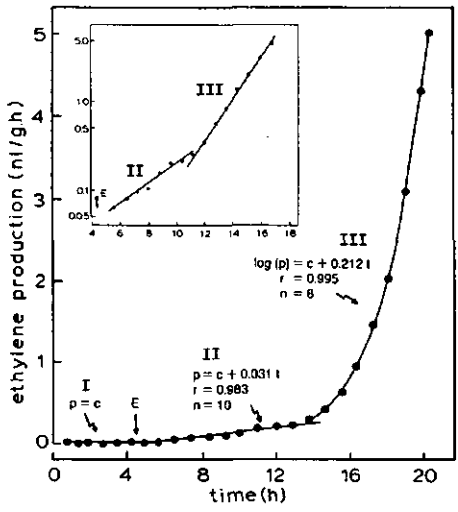

Fig. 3. Ethylene production (p) of *Phalaenopsis* cv. Red Lip plotted as a function of time after start of the experiment (t). E = time of emasculation. Insert: Ethylene production plotted on a logarithmic scale.

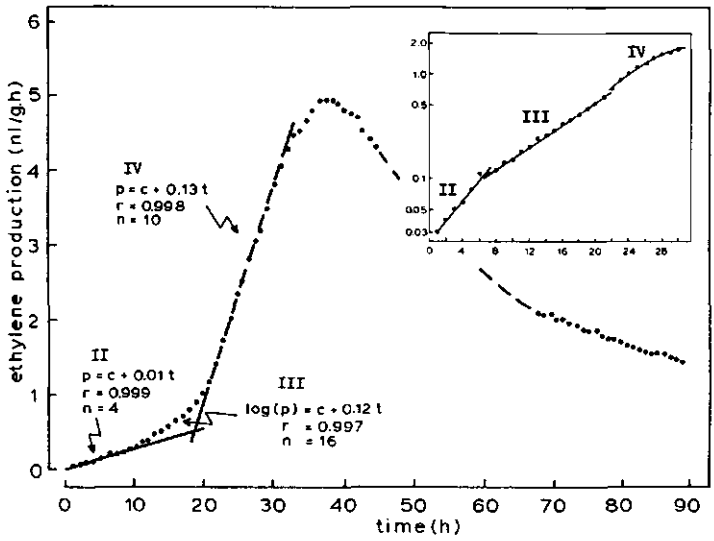

Fig. 4. Ethylene production (p) of *Epidendrum* (hybrid) as a function of time after emasculation (t). At t = 0 the flower was emasculated. Insert: Ethylene production plotted on a logarithmic scale.

In *Epidendrum* flowers (fig. 4) a linear increase in the ethylene production was observed starting within one h after emasculation (phase II). Between 5 and 20 h after emasculation the production showed a log-linear increase (phase III) and thereafter a linear increase was again apparent (phase IV). At about 35 h after emasculation the production reached its top (~ 5 nl/g.h) and showed a gradual decrease therafter.

In *Cymbidium* and *Oncidium* flowers a small peak in the ethylene production was observed within the first day after emasculation (Fig. 5). Thereafter the production stayed at a low level for some time. Coloration of the *Cymbidium* lip was observed at about 30 h after emasculation. At a later time again an increase in ethylene production was observed (data not shown).

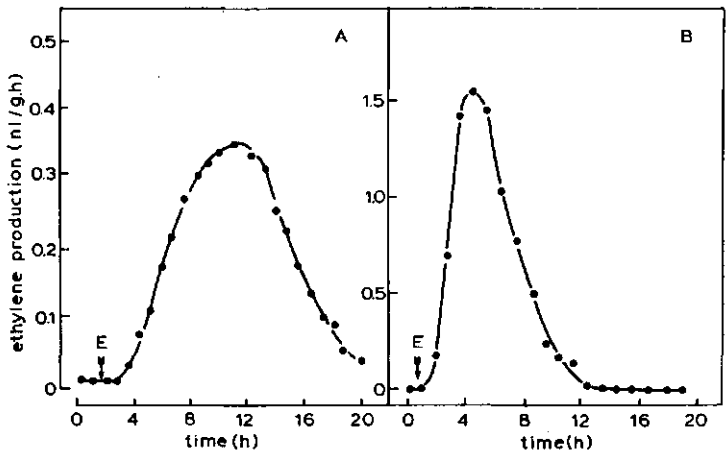

Fig. 5. Ethylene production of *Cymbidium* cv. King Arthur (A) and *Oncidium splendidum* (B) as a function of time. E = time of emasculation.

The effect of emasculation on membrane permeability (ion-leakage) of *Cymbidium* petal discs shown in figure 6. Within 24 h, the rate of $^{36}Cl^-$ efflux from petal discs of emasculated flowers was about two times greater than in non-emasculated flowers. This was also the case at 48 h after emasculation.

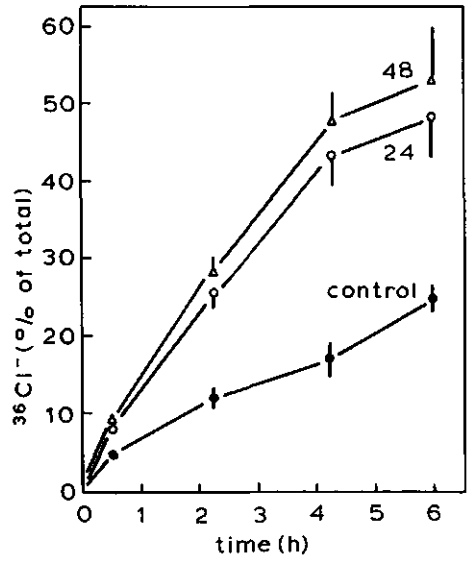

Fig. 6. Efflux of radioactive chloride from petal discs of *Cymbidium* cv. King Arthur. Flowers were left untreated (●) or emasculated at 48 (Δ) and 24 h (o) prior to start of the measurements. Values are means of 3 vials with 5 discs each. Vertical bars represent standard error, where s.e. bars were larger than symbols.

DISCUSSION

The laser-driven PA system used in our study was very sensitive for the determination of ethylene. The minimum detection limit in the present experiments was 0.03 nl/l, which is much lower than in any other method described so far. On-line detection of ethylene directly at the outlet of a continuous flow system with a single flower appeared possible without accompanying problems arising from an inadequate control of the gaseous composition in the cuvette. The measurements therefore represent a true estimate of the ethylene production in an undisturbed environment.

It was clearly shown that the sudden (log-linear) increase in ethylene production in carnation, emasculated *Phalaenopsis*, emasculated *Epidendrum* and non-emasculated *Cymbidium* flowers is preceded by a gradual increase of the basal production. In all cases, this small increase in production was better described by a linear relation than by a log-linear relation with time.

Although the pattern of ethylene production in emasculated *Cymbidium* and *Oncidium* flowers was quite different, it also consisted of clearly discernible phases *i.e.*, a small ethylene peak immediately after emasculation, a lag phase and thereafter a second, more pronounced increase. Thus, in all cases the increase in ethylene production which accompanies wilting of the flower was preceded by a small increase of the low, basal ethylene production. It is therefore suggested that this early increase in ethylene production initiates the more pronounced increase.

The conclusion that ethylene is not involved in the initiation of senescence in carnation (*e.g.* Eze *et al.*, 1986; Sylvestre and Paulin, 1987) may therefore be wrong because this conclusion was based on the observations that membrane properties started to change before the more pronounced rise in endogenous ethylene production. Our data show that the connection between ethylene production and senescence may be closer than can be derived from their results.

When carnations are pretreated with amino-oxyacetic acid (AOA; an inhibitor of ACC-synthase) the rise in ethylene production is considerably delayed (*e.g.* Borochov *et al.*, 1982; Woltering and Sterling, 1986). AOA has also been shown to alleviate the effect of drought stress which hastens the onset of the autocatalytic ethylene production in untreated flowers (Borochov *et al.*, 1982; Mayak *et al.*, 1985). This implies a role for ethylene in the initiation of senescence. Furthermore, treatment of carnations with exogenous ethylene induces both a rise in ethylene production and an increase in membrane permeability (*e.g.* Eze *et al.*, 1986; Sylvestre and Paulin, 1987).

Our data on ion-leakage from petals of emasculated *Cymbidium* flowers (Fig. 6) also clearly support the view that a very small change in ethylene production may already have a significant effect on membrane properties. The lack of correlation between the observed changes in the different parameters of cell senescence noted by various authors may therefore be at least partly due to the degree of sensitivity by which these parameters were measured.

Although we are now able to measure the ethylene production in a sensitive and accurate way, our experiments do not rule out the possibility that the observed early changes in ethylene production result from senescence-associated changes in the cell membranes. The production of free-radicals' associated with membrane deterioration, in turn, may stimulate ethylene production (Yang and Hoffman, 1984; Droillard *et al.*, 1987).

However, the data presented in this paper indicate that, as has been demonstrated for roses, ethylene may be the primary cause of senescence in carnation and other flowers as well. Carnations and orchids are generally much more sensitive to ethylene than roses and, therefore, minor changes in the low, basal ethylene production may already trigger senescence processes including the autocatalytic rise in ethylene production which in turn may be responsible for integration of the wilting process.

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APPENDIX

Basic principle of photoacoustic spectroscopy in gases

The photoacoustic (PA) effect is based on the generation of acoustic waves due to the deposition of heat in the sample following the absorption of energy. The effect was discovered by A.G. Bell (1881) on solids and was confirmed by Tyndall and Röntgen on aqueous and liquid samples.

An early photoacoustic set-up using the frame of an old sewing machine is shown in figure 1. The radiation from the sun was periodically interrupted by means of a mechanical chopper and directed into a small vessel containing a gaseous sample that absorbs in the infrared. Bell and contemporaries listened with a stethoscope connected to the vessel and perceived the feeble sound production at a frequency that corresponded to that at which the radiation source was modulated.

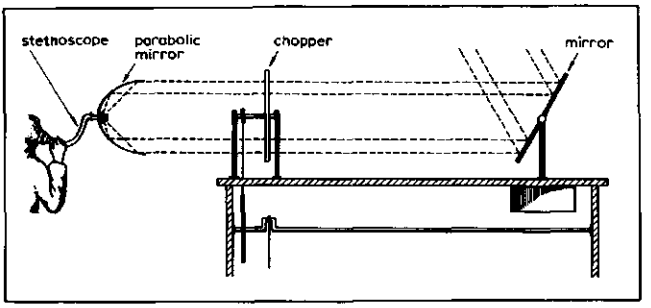

Fig. 1. Early photoacoustic detector (Bell, 1881).

Nowadays it is understood that this optically induced sound production results from the periodical absorption of the infrared portion of the energy provided by the sun. Following the absorption of energy, the gas molecules are excited from the ground state into the rotational level of a higher vibrational state and de-excitation processes will then redistribute the energy (Fig. 2). In the infrared region the probability for radiative decay is small and, in general, relaxation takes place along the non-radiative channel. For the majority of molecules the relaxation time at atmospheric pressure is fast (in the order of μs) as the rotational-translational de-excitation through collisions prevails. It causes an increase in kinetic energy of gas molecules and hence also of gas temperature.

Consequently, in a vessel of constant volume the increase in temperature leads to a corresponding increase in pressure. Thus, when the radiation source is modulated at audio frequency, the generated pressure changes can be detected by a microphone. The magnitude of the generated signal is proportional to the number of absorbing molecules (concentration) and the amount of power absorbed by the sample. In principle, regardless of the excitation beam (electro-magnetic radiation from very short to very long wavelengths, charged particles or other) the detection principle remains the same (Rosencwaig, 1980).

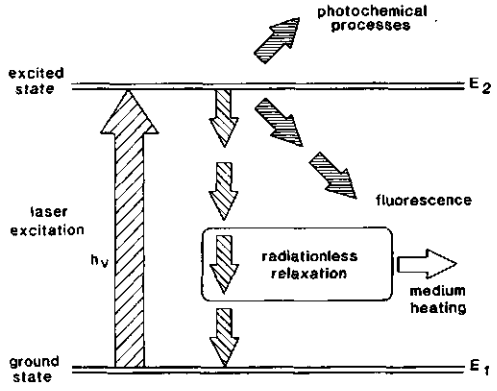

Fig. 2. Redistribution of energy on absorption of radiation from the excitation beam. Radiationless relaxation gives rise to a photoacoustic signal (Zharov and Letokhov, 1986).

Fifty years after Bell's discovery, the almost forgotten PA effect found its first application when Viengerov constructed an instrument to acoustically record spectra of N_2O. Later, concentration measurements were also performed (Viengerov, 1938). A revival of interest in photoacoustics was brought about by the advent of the laser. Soon after Kerr and Atwood (1968) demonstrated the usability of the laser to acoustically record the weak spectra of water vapor, Kreuzer (1971) used the He-Ne laser to investigate methane-nitrogen mixtures. When a high-power light source is used, the PA method becomes comparable or even superior to other spectroscopic techniques.

A true upsurge of interest has been noticed in the last decade, in particular for quantifying low concentrations of the atmospheric pollutants. In principle, more than 250 gases of environmental interest could be investigated with a CO_2 laser (Hubert, 1983).

Besides having a large number of other applications in agricultural, medical and other life sciences, in process engineering and areas of natural science, photoacoustic detection of photosynthetic activities has become a valuable tool in plant-physiological studies (*e.g.* Canaani and Malkin, 1984; Canaani, 1986). Recently we described a laser-driven photoacoustic system for measurement of low concentrations of ethylene in a continuous flow system (Woltering *et al.*, 1988). We used this system to establish whether changes in ethylene production may occur in advance of the autocatalytic upsurge during senescence of carnation and (emasculated) orchid flowers.

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CHAPTER 4

LIP COLORATION IN *CYMBIDIUM* FLOWERS BY EMASCULATION AND BY LIP-PRODUCED ETHYLENE

Ernst J. Woltering

SUMMARY

The effects of emasculation, exogenous ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC) and inhibitors of ethylene production and action on coloration of *Cymbidium* lips were investigated. Coloration of attached lips was dramatically hastened by emasculation and exogenous ethylene. Coloration of isolated lips was advanced by exogenous ethylene and ACC whereas amino-ethoxyvinylglycine, amino-oxyacetic acid and 2,5-norbornadiene delayed coloration considerably. The data therefore indicate that emasculation-induced coloration of the *Cymbidium* lip can be ascribed to the action of ethylene produced in the lip.

The nature of the signal linking emasculation and lip coloration is discussed.

Acta Horticulturae 261:145-150(1989).

INTRODUCTION

The first visible symptom of senescence in cut *Cymbidium* flowers is a color change of the labellum (lip) due to the accumulation of anthocyanins. This red coloration can be induced prematurely by various treatments including pollination, emasculation (removal of pollinia which includes removal of the anther cap) and by treatment of the flowers with exogenous ethylene or stylar application of indole-3-acetic acid (IAA) (Arditti *et al.*, 1973).

The overall effects of emasculation and ethylene treatment on these flowers were shown to be quite similar. Pollination and IAA, however, evoke additional post-pollination phenomena such as closing of the stigma and swelling of the gynostemium (Arditti *et al.*, 1973). As has been found in other orchids (Burg and Dijkman, 1967; Goh *et al.*, 1985; Nair *et al.*, 1984), emasculation of *Cymbidium* flowers was shown to advance the onset of the sudden upsurge in ethylene production that accompanies senescence in these flowers (Goh *et al.*, 1985).

In our own experiments (Woltering, 1990), emasculation-induced coloration of the *Cymbidium* lip appears several days before the start of this rise in ethylene production suggesting that these two events are not directly related. Indeed, it has recently been shown that coloration is preceded by a much smaller peak in endogenous ethylene production starting at about 3 h after emasculation (Woltering *et al.*, 1988).

This paper discusses the effect of exogenous ethylene and of various chemicals known to suppress or evoke ethylene effects, on coloration of *Cymbidium* lips.

MATERIALS AND METHODS

Cymbidium flower spikes (cv. King Arthur) were obtained from commercial growers and transported dry to the laboratory. The experiments were conducted with groups of individual flowers or isolated lips cut from the middle region of the flower spike. During the treatments, the flowers and isolated lips were held with their cut bases in distilled water.

Treatment of individual flowers and isolated lips with ethylene and 2,5-norbornadiene (NBD) was carried out in stainless steel plant chambers at 20 ± 1°C in darkness. During the treatments, the carbon dioxide concentration was kept low (< 0.05%; v/v) and the oxygen concentration was maintained at am-

bient level (Woltering and Sterling, 1986).

Ethylene concentrations (0.03 to 0.3 μl/l) were obtained by injection of a known amount of ethylene gas (Matheson, Oevel, Belgium) into the chambers. An NBD concentration of ca. 2000 μl/l (v/v) as a gas was obtained by injection of a known amount of liquid NBD (Aldrich-chemie, Steinheim, West Germany) into the chamber. After the treatment (if relevant) the product was placed under controlled environmental conditions of 12 h white light (15 μmol/m^2.s) and 12 h darkness; 60% relative humidity and 20°C.

Isolated lips were treated with amino-oxyacetic acid (AOA; Sigma, St. Louis, USA); aminoethoxyvinylglycine (AVG; Sigma) and 1-aminocyclopropane-1-carboxylic acid (ACC; Sigma) by placing the lips with their cut base in an aqueous solution of the chemicals.

Red coloration of the lips was classified on a scale from 0 to 4, with a discrimination of about 0.5, using color prints as follows: 0 = original color; 1 = slightly pink coloration; 2 = pink coloration; 3 = red coloration and 4 = dark-red coloration.

All experiments (except for the continuous treatment and the treatment lasting 24 h in the plant chambers) were carried out under controlled environmental conditions as noted above. The experiments were carried out with 10 flowers or lips per treatment and were repeated at least once. Representative data are shown.

RESULTS

The time course of lip coloration following emasculation is shown in figure 1. Within 30 h after emasculation the lip showed a slightly pink coloration that increased in intensity thereafter to dark red after about 3 days. Lips from control flowers did not show a change in color during the experimental period.

The effect of a 24 h treatment with exogenous ethylene is shown in figure 2. At a low concentration, coloration of both attached and isolated lips was less intense than at higher ethylene concentrations. After initial ethylene-induced coloration, the intensity did not show marked changes until the start of coloration of the control flowers or lips (0 μl/l ethylene). The number of days to visible coloration of isolated control lips was about twice that of lips attached to the control flowers. The effect of ethylene on coloration of the lips, however, was essentially the same, showing a response above 0.1 μl/l (Fig. 3).

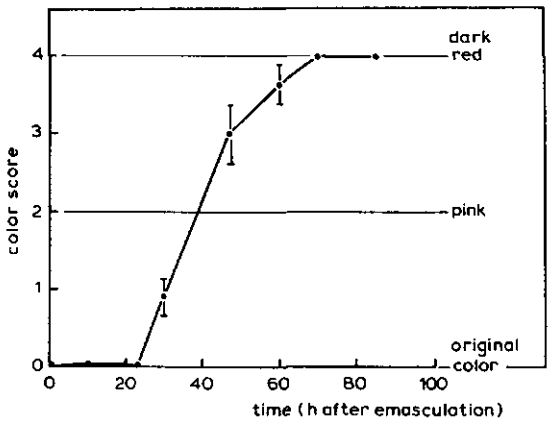

Fig. 1. Development of lip coloration in *Cymbidium* flowers (cv. King Arthur) after emasculation (n=10). Vertical bars represent 2 x s.d.

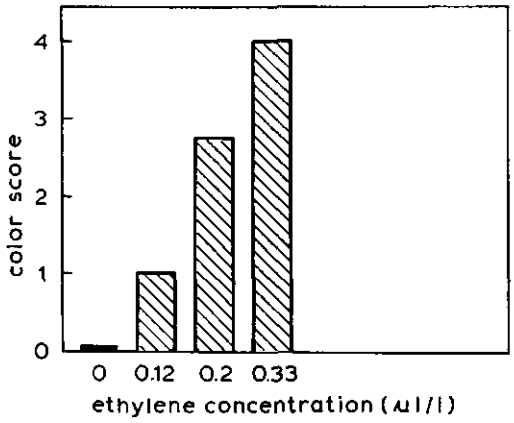

Fig. 2. Effect of ethylene (24 h at 20°C) on color intensity of attached and isolated *Cymbidium* lips (cv. King Arthur) (n=20).

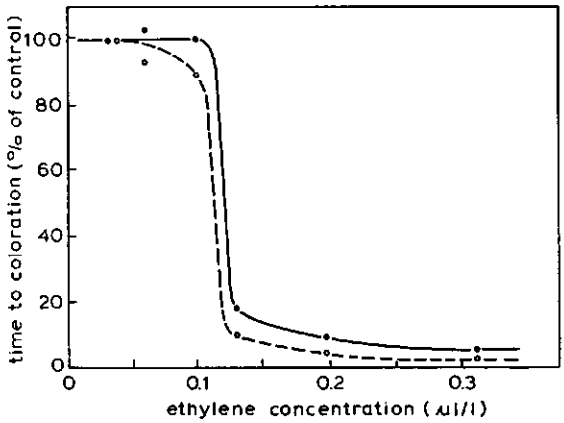

Fig. 3. Effect of ethylene (24 h at 20°C) on coloration of attached (——) and isolated (---) lips of *Cymbidium* flowers (cv. King Arthur) (n=10). Time to coloration (color score ≥ 1) of attached and isolated control lips was 11 and 20 days, respectively.

The effect of various chemicals on coloration of isolated lips is summarized in table 1. After 40 days the experiment was terminated because of the appearance of fungi on the lips treated continuously with NBD.

Continuous treatment of the lips with AVG or AOA delayed coloration whereas treatment with ACC dramatically hastened it. Treatment of the lips with ethylene and NBD showed that coloration was considerably delayed in the presence of NBD while even the effect of continuous treatment with a high concentration of ethylene was nullified by the NBD.

Table 1: Effect of various chemicals on coloration (color score ≥ 3) of isolated *Cymbidium* lips (n = 10)

chemical used	no. of days to coloration ± s.d.
water	22 ± 0.9
AOA (0.1 mmol/l)	27 ± 1.9
AVG (0.1 mmol/l)	> 40
ACC (0.05 mmol/l)	< 1
Air	24 ± 2.3
Ethylene (1 µl/l)	< 1
NBD (2000 µl/l)	> 40
Ethylene (1 µl/l) + NBD (2000 µl/l)	> 40

DISCUSSION

Red coloration of both attached and isolated *Cymbidium* lips was shown to be stimulated in a comparable way by treatment with low concentrations of ethylene (Figs. 2 and 3). The effect of ethylene thus seems to be a direct effect on the processes in the lip and does not involve regulation by the rest of the flower.

At low ethylene concentrations this coloration is less intense than at higher concentrations and upon removal of the ethylene the coloration process is hampered (data not shown), indicating that the process requires a certain ethylene dose and not just stimulation of an endogenous autonomous process.

Placing isolated lips in low concentrations of ACC evokes an increased ethylene production (data not shown) and rapid coloration. Inhibitors of ethylene production (AVG, AOA) and of ethylene action (NBD), however, were shown to delay coloration considerably (Table 1). The data therefore indicate that coloration of *Cymbidium* lips is an effect due to the action of locally produced ethylene. Theoretically the ethylene response in the lip may involve an increase in ethylene production, an increase in ethylene sensitivity or a combination of both.

In carnation it was shown that there is a rapid increase in ethylene production and ACC content in all the flower parts following pollination (Nichols *et al.*, 1983) and it was demonstrated that ACC, applied to the stigma, could be transported to the petals (Reid *et al.*, 1984). Besides this flow of ACC it has recently been suggested that in various flowers also a second signal, *i.e.* an ethylene-sensitivity factor, is involved in pollination-induced corolla senescence (Halevy *et al.*, 1984, Halevy, 1986).

Lip coloration upon emasculation (Fig. 1) may also be regulated by ethylene. It is of interest which signal, evoked by a treatment that operates at the tip of the central column, is responsible for the rapid ethylene response in a flower part at several cm distance.

The experiments presented in this paper show that the *Cymbidium* flower may offer a suitable model for investigation of the possible involvement of different factors in senescence. In these flowers a rapid ethylene response (that is easy to assess visually) can be evoked in the lip in a reproducible way by removal of the pollinia. Furthermore, no exogenous substances from the pollen (*e.g.* auxins, ACC) are involved, which makes interpretation of the results less complicated.

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CHAPTER 5

REGULATION OF ANTHOCYANIN SYNTHESIS IN *CYMBIDIUM* FLOWERS: EFFECTS OF EMASCULATION AND ETHYLENE

Ernst J. Woltering and Dianne Somhorst

SUMMARY

Emasculation, by removal of the pollinia and the anther cap, and treatment with ethylene induced red coloration of *Cymbidium* lips. This coloration was shown to be a result of anthocyanin accumulation that was preceded by a pronounced increase in the activity of phenylalanine ammonia-lyase. Ethylene-induced coloration of isolated lips was inhibited by treatment with 2,5-norbornadiene, cycloheximide and, to a lesser extent, by actinomycin-D. The results, therefore, indicate that emasculation-induced and ethylene-induced anthocyanin synthesis is regulated both at the translational and transcriptional levels.

The red color of the lip was composed of at least six anthocyanin species that were based on three different aglucones *i.e.* cyanidin, peonidin and malvidin. Emasculation and ethylene both led to an increase in the total amount of pigment in the lip but had no effect on the relative contribution of the different anthocyanin species. The role of red coloration in pollination biology is discussed.

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INTRODUCTION

In cut *Cymbidium* flowers the first visible sign of senescence is a severe red coloration of originally non-colored regions in the labellum (lip) due to the accumulation of anthocyanins. Pollination as well as emasculation by removal of the pollinia and the anther cap were shown to dramatically hasten the incidence of lip coloration and wilting of the perianth (Arditti *et al.*, 1973). A similar change of color is apparent upon pollination of for instance *Lantana* and *Lupinus* flowers (Wainwright, 1978; Mohan Ram and Mathur, 1984). Treatment of whole *Cymbidium* flowers or isolated lips with ethylene was also shown to induce coloration and the effect of ethylene on coloration of isolated lips was effectively counteracted by simultaneous treatment with the inhibitor of ethylene action, 2,5-norbornadiene (NBD) (Arditti *et al.*, 1973; Woltering, 1989).

Following emasculation, the *Cymbidium* flower initially produces a small peak in ethylene production after which coloration becomes visible (Woltering *et al.*, 1988). It may therefore be suggested that at least part of the ethylene is produced by the lip and this, in turn, causes lip coloration.

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) has been widely recognized as a key enzyme in general phenylpropanoid metabolism, including the synthesis of anthocyanins (Hahlbrock *et al.*, 1980). Ethylene has been shown to stimulate PAL-activity in a number of different systems (Hyodo and Yang, 1971; Chalutz, 1973; Chalmers and Faragher, 1977).

The use of orchids as ornamentals is largely due to the size and colors of the flowers. However, relatively little is known about the nature and distribution of their anthocyanins (Arditti and Fisch, 1977). Sugiyama *et al.*, (1977) demonstrated the occurrence of two anthocyanins in a number of different *Cymbidium* species. One was identified as cyanidin-3-glucoside, the other as cyanidin-3-diglucoside. The former had previously been isolated from *Cymbidium finlaysonianum* by Lowry and Keong (1973). To our knowledge, however, no detailed analysis of the anthocyanin composition in *Cymbidium* has been reported to date.

This paper reports on the effects of exogenous ethylene and emasculation on PAL-activity and anthocyanin synthesis in the *Cymbidium* lip. In addition, the anthocyanin composition in three different cultivars was analysed by reversed-phase high performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Plant material and coloration studies

Inflorescences of *Cymbidium* cv. Jacobi were obtained from a commercial grower and transported dry to the laboratory. The experiments were conducted with groups of individual flowers or isolated lips cut from the middle region of the spike. Generally, after excision the flowers or isolated lips were placed with their cut base in water under controlled environmental conditions of continuous white light (15 $\mu mol/m^2.s$), 60% relative humidity and 20°C.

Red coloration of the lips was visually judged and classified on a scale from 0 to 4 with a discrimination of about 0.5 using color prints as follows: 0 = original color; 1 = slightly pink coloration; 2 = pink coloration; 3 = red coloration and 4 = dark-red coloration (Woltering, 1989).

Ethylene and NBD treatments

Treatment of individual flowers and isolated lips with ethylene and NBD was carried out in stainless steel plant chambers (contents 70 L) at 20 ± 1°C in darkness. During the treatments, the carbon dioxide concentration was kept low (< 0.05%, v/v) and the oxygen concentration was maintained at ambient level. Details of the system have been described by Woltering and Sterling (1986). An ethylene concentration of 3 $\mu l/l$ (v/v) was obtained by injection of a known amount of ethylene gas (UCAR Specialty Gases, Oevel, Belgium) into the chambers. An NBD concentration of ca. 2000 $\mu l/l$ (v/v) as a gas was obtained by injection of a known amount of liquid NBD (Aldrich-chemie, Steinheim, West Germany) into the chamber.

Treatment of isolated lips with chemicals

The isolated lips were treated with cycloheximide (CHX; Sigma, St. Louis, USA) and Actinomycin-D (Act-D; Sigma, St. Louis, USA) by placing the lips with their cut bases in aqueous solutions of the chemicals. After approximately 24 h the lips, still in the pretreatment solutions, were treated with ethylene as described above. At the end of the ethylene treatment, one group of lips was immediately used for the analysis of PAL-activity, the others were transferred to water to study the development of red coloration.

Analysis of total anthocyanins

At different times after emasculation or treatment of the flowers with ethylene, 10 to 20 lips were excised, frozen in liquid nitrogen and lyophilized. Dry matter was ground in a mini ball-mill (Retsch MM2, Ochten, The Netherlands) and 50 mg powder was extracted for 24 h at 5°C with 5 ml 80% methanol: 1% HCl (v/v). Absorbance was measured spectrophotometrically at 520 nm (Uvicon). Total anthocyanins were expressed as mg/ml cyanidin using a calibration curve of authentic cyanidin (Roth, Karlsruhe, West Germany) dissolved in 80% methanol : 1% HCl (v/v).

Determination of PAL-activity

For PAL determination, 50 mg powder was homogenized for ca. one min in 5 ml 100 mM cold borate buffer (pH 8.8) containing 5 μl β-mercaptoethanol. After centrifugation for 10 min at 15,000 g and 2 °C the supernatant was used for further analysis.

The determination of PAL-activity was carried out as described by Legrand *et al.*, (1976). The reaction mixture consisted of 0.4 ml supernatant, 1.4 ml 100 mM borate buffer (pH 8.8) and 0.1 ml of the same buffer containing 1850 Bq of [U-^{14}C] L-phenylalanine and 2 mM L-phenylalanine. After incubation for 2 h at 37°C the reaction was stopped by adding 0.1 ml 5 N HCl. Cinnamic acid was extracted by addition of 10 ml of an ether-cyclohexane (1:1) mixture. After shaking for ca. 5 min, the vials were centrifugated for ca. 1 min at 2,000 g, and thereafter a 5 ml sample of the organic phase was allowed to evaporate and the radioactivity in the residue was measured by liquid scintillation counting.

The background level of radioactivity due to the contamination of the extracted cinnamic acid by traces of labeled phenylalanine was determined in a reaction mixture that was treated as described above except that the reaction was stopped at time zero. PAL-activity was expressed as the amount of cinnamic acid formed by 1 ml supernatant in one h.

Separation and identification of anthocyanins

Anthocyanins in lips were extracted for 24 h from dried tissue with an aqueous 1% (v/v) HCl solution. Afer centrifugation a sample of the extract (10 to 20 μl) was analysed by HPLC (Wilkinson *et al.*, 1977). The equipment consisted of two Waters Model 6000 A pumps and a Waters Model 660 solvent programmer. The HPLC was equipped with a Porapak C18 reversed-phase column and a Waters Model 990 diode array detector (Hebrero *et al.*, 1988).

Separation of the pigments was accomplished by gradient elution using a 10% formic acid solution (solvent A) and acetonitril (solvent B). The gradient used for separation of anthocyanins was non-linear, from 10% B in A to 55% B in A with a flow rate of 0.6 ml/min. Anthocyanidins were determined after acid hydrolysis of the extract for 2 h at 100°C. In this case a non-linear gradient of 10% solvent B in A to 100% solvent B in 45 min with a flow rate of 0.6 ml/min was used.

Anthocyanidins were characterized by retention time and spectral properties. Identification was accomplished by HPLC co-chromatography with authentic substances (Apin Chemicals Ltd., Abingdon, U.K.).

RESULTS

Coloration of lips

The development of red coloration as determined by visual inspection is shown in figure 1. Both in lips from emasculated and from ethylene-treated flowers a faint pink coloration in the originally non-colored regions appeared within 24 h of treatment and this developed into dark red within the following day. In control flowers no change in color appeared during the first days of the experiment. The development of coloration was similar in light or dark (Fig. 1).

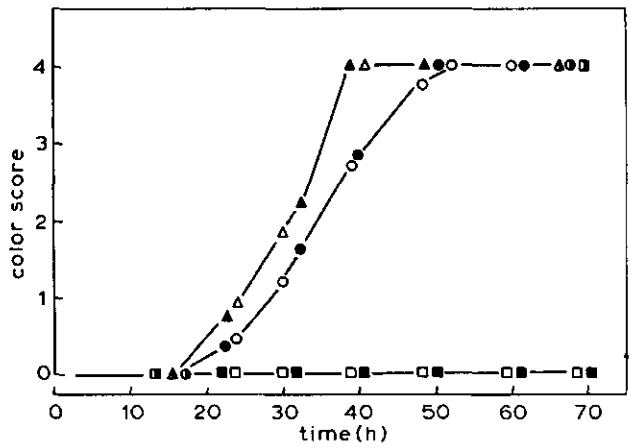

Fig. 1. Development of red coloration in *Cymbidium* lips as determined by visual inspection (see M + M). Flowers were emasculated (o,●), treated with 1 µl/l ethylene (△,▲) or left untreated (□,■). Solid symbols represent data from flowers treated in the dark, open symbols from flowers treated in continuous light (n=10).

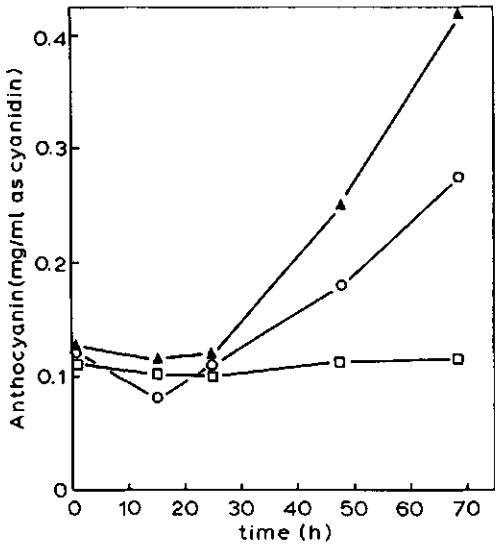

Fig. 2. Accumulation of anthocyanins in lips.
Flowers were emasculated (o), treated with 1 μl/l ethylene (▲) or left untreated (□). Standard errors were less than 3 % of the mean (n=3).

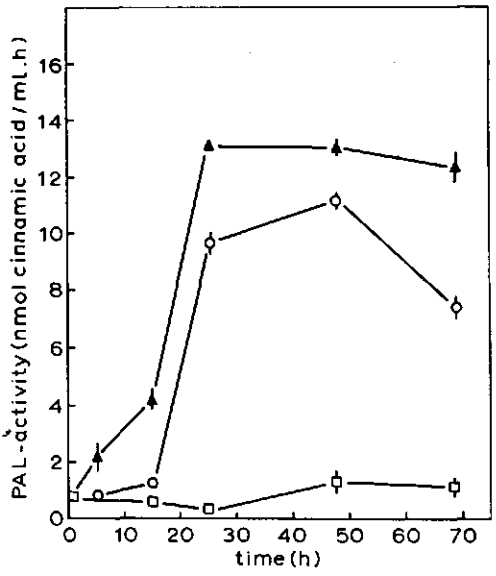

Fig. 3. Development of PAL-activity in lips.
Flowers were emasculated (o), treated with 1 μl/l ethylene (▲) or left untreated (□). Values are means of 3 determinations. Vertical bars represent 2 x s.e., where s.e. bars were larger than symbols.

The contents of anthocyanin in lips as determined spectrophotometrically showed the same trend as coloration observed by visual inspection (Fig. 2). Due to the high background level as a result of anthocyanins present in the originally dark-red pigmentated regions, the overall increase in anthocyanin became measurable at a time when it was already clearly noticeable by visual inspection. On the other hand, the anthocyanin content continued to increase at a time when no further discrimination could be made by visual inspection.

PAL-activity

The changes in PAL-activity are shown in figure 3. Both in lips from emasculated and from ethylene-treated flowers a sharp increase in activity was apparent within 24 h of treatment and this activity remained high during the following days. In lips from control flowers no significant change in PAL-activity was observed during the experimental period.

The effect of different chemicals on ethylene-induced PAL-activity and coloration of isolated lips is shown in table 1. CHX and NBD completely inhibited both the coloration and the rise in PAL-activity, whereas Act-D was only partially effective.

Table 1. Effect of different chemical treatments on ethylene-induced coloration and changes in PAL-activity in isolated lips. The lips were placed in water, CHX or Act-D 24 h prior to treatment with ethylene.
PAL-activity (means of three determinations ± s.d.) was determined immediately following the ethylene treatment and expressed as a percentage relative to the activity in ethylene-treated control lips (fixed at 100 %). Time to coloration (means of 10 lips ± s.d.) represents the visual determination up to color score 3.

Treatment	Time to coloration (h)	PAL-activity (%)
Air	> 72	11 ± 0.4
Air + NBD (2000 µl/l)	> 72	n.d.[1]
Air + CHX (1.0 mmol/l)	> 72	n.d.
Air + Act-D (0.5 mmol/l)	> 72	n.d.
Ethylene (1 µl/l)	38 ± 8	100 ± 4.0
Ethylene + NBD	> 72	8 ± 0.2
Ethylene + CHX	> 72	13 ± 1.4
Ethylene + Act-D	> 72[2]	55 ± 3.1

[1] n.d. = not determined
[2] a slight pink coloration appeared (color score 1 to 2); it did not develop into red

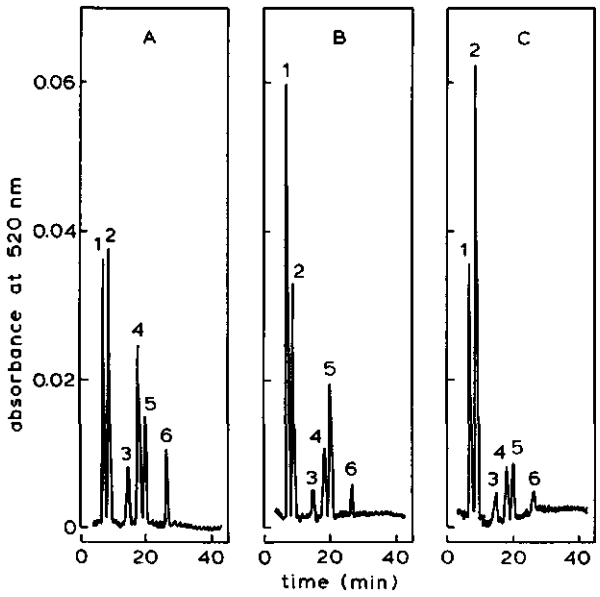

Fig. 4. HPLC chromatograms of anthocyanins in lips from three different *Cymbidium* cultivars. In all cases the same amount of pigment, as measured by the overall absorbance at 520 nm, was injected.
A = cv. Jacobi; B = cv. King Arthur; C = cv. Ivy Fung Sultan.

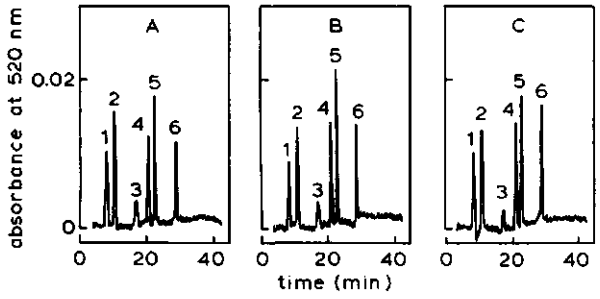

Fig. 5. HPLC chromatograms of anthocyanins in lips from control (A), emasculated (B) and ethylene-treated (C) *Cymbidium* flowers cv. Jacobi. In all cases the same amount of pigment, as measured by the overall absorbance at 520 nm, was injected.

Anthocyanin composition

On the HPLC chromatograms of anthocyanins six major peaks were present and in some cases a seventh, smaller one. In the three different cv. tested, these main peaks were detected although the relative contribution of the individual components to the total amount differed somewhat (Fig. 4). In addition it was established that the anthocyanin composition in groups of flowers from the same cv. but harvested at different times may vary considerably (compare Figs. 4A and 5A). To investigate whether the changes in color induced by emasculation and ethylene were due to the induction of the same set of anthocyanins as originally present in the lip, samples of the differently treated flowers, with the same overall absorption at 520 nm, were compared. Both emasculation and treatment with ethylene increased the total amount of anthocyanins whereas the relative contribution of the different anthocyanin species was hardly changed (Fig. 5).

Following acid hydrolysis, only 3 peaks were detected indicating that the anthocyanins found were based on 3 different aglucones. By co-chromatography on HPLC the aglucones were identified as cyanidin, peonidin, and malvidin.

DISCUSSION

The induction of red coloration following emasculation or treatment with ethylene was not affected by light and was shown to involve a pronounced increase in anthocyanin content. Well before the start of the increase in anthocyanin level, an increase in PAL-activity was observed.

Changes in PAL-activity have been described in response to various types of stress, including microbial attack, wounding and treatment of the tissue with ozone or ethylene (Koukol and Dugger, 1967; Tena *et al.*, 1984; Érsek and Király, 1986). The close correlation between the effect of emasculation and exogenous ethylene on PAL-activity and anthocyanin accumulation indicates that ethylene is the sole regulator of anthocyanin synthesis during senescence of these flowers. The effect of the gaseous inhibitor of ethylene action, NBD, on ethylene-induced coloration confirms this view. The inhibitory effects of CHX and Act-D on anthocyanin synthesis and PAL-activity indicates that the enzyme was *de novo* synthesized in response to ethylene and that both transcription and translation processes may be involved.

The red color of the lip is composed of at least six different anthocyanin species that are based on three different aglucones. In the tested cultivars

emasculation or treatment with ethylene did not significantly alter the relative contribution of the different anthocyanins to the color of the lip. This shows that the biochemical steps leading to the synthesis of specific anthocyanin species are the same in all stages of development and that one single stimulus, *i.e.* ethylene, may regulate them all. A comparable phenomenon has been described by Hahlbrock *et al.* (1980). These authors observed a simultaneous increase in the activity of a variety of enzymes involved in anthocyanin synthesis in cell-cultures upon UV-irradiation.

In *Lantana* flowers, which are normally pollinated by thrips, it was found that the pollinators were only attracted by yellow flowers. The change of color following pollination may therefore serve as a mechanism to preserve pollinator energy (Mathur and Mohan Ram, 1978; Mohan Ram and Mathur, 1984). Similarly, the post-pollination change in color of the so-called "banner spot" from yellow-white to deep purplish-red in *Lupinus* flowers was shown to re-direct a variety of pollinators to the other, non-pollinated flowers (Wainwright, 1978). The reason may be that most insects are considered to be red-blind and especially attracted by yellow patches (Goldsmith and Bernard, 1974; Kay, 1976).

The biological role of pollination or emasculation-induced coloration of the *Cymbidium* lip may also be considered as a way to re-direct pollinators, mainly bumble bees (*Bombus* sp.). In many orchids, parts of the central column and the lip were shown to possess stronger ultraviolet light absorption than the other floral appendages. As many insects are capable of detecting ultraviolet radiation this may help them in finding the nectar thereby favouring pollination (Thien, 1971). The change in color that appears in the lip and also in the central column may change the overall pattern of light reflection making the flower less attractive to pollinators. In case of the emasculated flower the rapid change in color seems most undesirable as these flowers have not yet been pollinated. Apparently, the flower can be abandoned after having functioned as a pollen donor.

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CHAPTER 6

ROLE OF ROSTELLUM DESICCATION IN EMASCULATION-INDUCED PHENOMENA IN ORCHID FLOWERS

Ernst J. Woltering and Frans Harren

SUMMARY

The effects of the relative humidity (RH) and of treatment of the rostellum with aminoethoxyvinylglycine (AVG), aminocyclopropane-1-carboxylic acid (ACC), and water-insoluble vacuum grease on emasculation-induced phenomena in *Cymbidium* and *Phalaenopsis* flowers were investigated.

Under conditions of high RH and after treatment of the rostellum with water-insoluble grease, the normal response to emasculation (*i.e.* increased ethylene production, lip coloration and wilting) was absent. However, under conditions of high RH this response could be restored by the addition of 2.0 nmol ACC onto the rostellar surface. Under conditions of low RH the response was inhibited by AVG; this inhibition was partially reversed by addition of 2.0 nmol ACC.

The data provide evidence that desiccation of the rostellum is responsible for post-emasculation phenomena in orchid flowers.

Journal of Experimental Botany 40:907-912(1989).

INTRODUCTION

In orchids, the male structures are composed of 2, 4, 6 or 8 pollinia that are positioned on the top of the gynostemium (central column) which itself represents a fusion of stamens (anthers and filaments), stigmas and styles (Arditti, 1979).

The pollinia of most orchids have a stalk or plate-like tissue (called the pollinium stalk) by which they are connected to the viscidium which is an integral part of the rostellum (Rasmussen, 1986), the latter being an organ which is supposed to be of stigmatic origin (Arditti and Flick, 1974). In many orchids, the pollinia and at least part of the rostellum are covered by an anther-derived tissue known as "anther cap". Both pollinia and anther cap are only loosely connected to the central column and they may be simultaneously removed by a visiting insect, thereby uncovering the rostellum.

Removal of pollinia and anther cap (emasculation) has been shown to hasten the sudden upsurge in ethylene production which accompanies senescence in various orchid species (Burg and Dijkman, 1967; Nair *et al.*, 1984; Goh *et al.* 1985). In *Cymbidium*, it was found that the effects of emasculation were very similar to those of treatment of the flowers with exogenous ethylene, *i.e.* an increased synthesis of anthocyanins in the central column and the lip and advanced wilting of the flower (Arditti *et al.*, 1973). Pollination and stylar application of auxin, however, were shown to evoke additional post-pollination phenomena such as closing of the stigma and swelling of the gynostemium (Arditti *et al.*, 1973). Hence, it was suggested that the rostellum is involved in ethylene-associated phenomena and that it may be the site of ethylene production in pollinated orchid flowers (Arditti and Flick, 1974).

On removal of the pollinia, the viscidium also comes off as a sticky pad. It has therefore been suggested that mechanical effects on the rostellum are the cause of subsequent physiological activity (Arditti and Flick, 1974). Van Staden (1979), in contrast, suggested that the high levels of endogenous cytokinins present in the pollinia and anther cap of *Cymbidium* flowers may be involved in prevention of the onset of senescence, either by creating a "sink" for water and nutrients or by inhibition of ethylene production.

In an attempt to elucidate the role of the rostellum, we investigated the effect of various conditions and of chemicals that are known to suppress or stimulate ethylene production (Yang and Hoffman, 1984), on emasculation-induced ethylene production and senescence symptoms in *Cymbidium* and *Phalaenopsis* orchids.

MATERIALS AND METHODS

Plant material

Inflorescences of *Cymbidium* cv. King Arthur and *Phalaenopsis* cv. Red Lip were obtained from commercial growers and transported dry to the laboratory. The experiments were carried out with individual flowers cut from the middle region of the spike. After excision, the flowers were individually placed in 10 ml tubes containing tap water.

Vase-life studies

The experiments were carried out under controlled environmental conditions of 12 h white light (15 $\mu mol/m^2.s$) and 12 h darkness; 60% relative humidity (RH) and 20°C throughout.

For high RH, the flowers were placed in a 25 L desiccator (containing ca. 1 l water) which was continuously flushed with humidified air. To obtain a locally high or low RH at the rostellar region, a small tube (0.7 x 10 cm) containing either wet cotton wool or a few grains of $CaCl_2$ was positioned over the central column.

The development of senescence symptoms was judged visually each day. In *Phalaenopsis*, the average number of days to wilting and in *Cymbidium*, the average number of days to coloration (color score ≥ 3) of the lip (being the first visible sign of senescence) was calculated.

Red coloration of the *Cymbidium* lips was classified on a scale from 0 to 4 with a discrimination of about 0.5, as follows: 0 = original color; 1 = slightly pink coloration; 2 = pink coloration; 3 = red coloration; 4 = dark-red coloration (Woltering, 1989).

Treatment of the flowers with chemicals

The flowers were treated with aqueous solutions of aminoethoxyvinylglycine (AVG; Sigma) and 1-aminocyclopropane-1-carboxylic acid (ACC; Sigma) as a small droplet (1.0, 2.0 or 10.0 μl) applied to the rostellar surface immediately after emasculation. When ACC was applied to a flower that had previously been treated with AVG this was done at about 20 h after the AVG treatment.

In some experiments, approximately 50 mg of water-insoluble grease ("Glisseal"; Borer Chemie A.G., Solothurn, Zwitserland) was smeared onto the rostellum immediately after emasculation.

Measurement of ethylene production

Ethylene evolution of individual flowers was measured at 21°C using photoacoustic equipment that consisted of a line-tuneable CO_2 laser in conjunction with a single-pass resonant photoacoustic cell (Woltering, *et al.* 1988). The minimum detection limit of the detector for ethylene was approximately 0.03 nl/l. The flowers were placed in small glass cuvettes (contents 80 ml) that were continuously flushed with purified air (< 0.5 nl/l ethylene) at a flow rate of 0.9 l/h to prevent accumulation of carbon dioxide, ethylene and water vapor. The effluent of the cuvette was directed through the photoacoustic cell for determination of ethylene.

To obtain a high and a low RH inside the cuvettes the air was humidified (bottle of water) or dried (column with $CaCl_2$ grains) prior to entering the cuvettes, and a small amount of water or $CaCl_2$ was placed in the cuvettes. The RH inside the cuvettes was measured with a "Murata" dew-point sensor. The emasculation treatment and the application of chemicals to the rostellum were carried out through a septum-stoppered port in the cuvette, thereby avoiding any alteration to the gas composition inside the cuvette.

All experiments were repeated at least once and representative data are shown.

RESULTS

Effect of rostellum desiccation on senescence symptoms

At a low RH (~ 60%), emasculation caused rapid coloration (within approximately 1.5 d) of *Cymbidium* lips and advanced wilting both in *Cymbidium* and in *Phalaenopsis* flowers. In both genera, however, this response to emasculation was almost completely absent in a humid (98 to 100% RH) environment (Table 1). The humid atmosphere will delay or inhibit desiccation of the uncovered rostellum. From the experiments in which the central column of the emasculated flower was covered by a "wet" or a "dry" capillary tube it was shown that the central column and not the whole flower is in fact the site of the effect of different humidity levels in the surrounding atmosphere (Table 1).

If *Cymbidium* flowers were emasculated in a humid environment and later on transferred to conditions with a much lower RH the lip showed rapid coloration comparable to flowers emasculated under dry conditions, indicating that

the process is initiated by the low humidity and not by the emasculation itself (Fig. 1). When water-insoluble grease was smeared onto the rostellum immediately after emasculation, the response was considerably delayed in *Phalaenopsis* and even completely absent in *Cymbidium* (Table 1). This also indicates that desiccation of the rostellum is responsible for emasculation-induced phenomena.

Table 1. Effects of various treatments on lip coloration (color score ≥ 3) in *Cymbidium* and on wilting in *Phalaenopsis* at low RH (~ 60%) and high RH (~ 100%) (n=5). Grease and aqueous solutions of the chemicals were applied to the rostellum; the capillary tube was placed over the upper half of the central column (see M + M).

Treatment	Time to lip coloration in *Cymbidium* (days)	Time to wilting in *Phalaenopsis* (days)
Low humidity		
Intact	10	6.5
Emasculation (E)	1.5	2.5
E + Water	1.5	2.5
E + AVG (10.0 nmol)	18	5
E + AVG (10.0 nmol) + ACC (2.0 nmol)	1	5
E + Grease	12	5.5
E + Wet capillary tube	7	6
High humidity		
Intact	11	10
Emasculation (E)	12	7
E + Water	10	8
E + ACC (2.0 nmol)	2	2
E + Dry capillary tube	3	3
LSD (0.05)	2.6	0.9

Treatment of the rostellum with AVG and ACC

Under conditions in which the rostellum was allowed to desiccate (low humidity) but a small amount of AVG (10.0 nmol) was applied to its surface, the response to emasculation was greatly suppressed in both genera. When a small amount of ACC (2.0 nmol) was added to the AVG-treated rostellum, the coloration response in *Cymbidium* could be restored, whereas ACC had no stimulatory effect on wilting in *Phalaenopsis* (Table 1).

Under humid conditions, the addition of a small amount of ACC restored the normal response to emasculation in both genera (Table 1). These data indicate that the response to desiccation is mediated by ACC or ethylene.

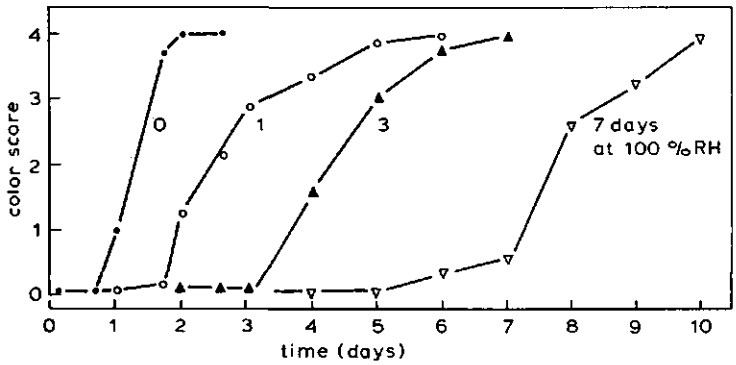

Fig. 1. Development of red coloration (see M + M) of *Cymbidium* lips after emasculation.
Flowers were emasculated under conditions of high RH (~ 100%) and subsequently transferred to conditions of low RH (~ 60%) after zero (•); one (o); three (▲) and seven (▽) days (n = 7).

Effect of rostellum desiccation on ethylene production

The pattern of ethylene production following emasculation at low RH was quite different in these two genera (see also Woltering *et al.*, 1989). In *Cymbidium* there was a small peak in ethylene production starting a few hours after emasculation (Fig. 2). At about 24 h after emasculation, the lip showed the first visible sign of coloration. Depending on the other conditions, one to several days later a much more pronounced upsurge in ethylene production appeared (with peak values of 1.5 to 2 nl/g.h) accompanying senescence (data not shown).

In *Phalaenopsis*, the ethylene production showed a gradual increase starting almost immediately after emasculation. About 12 h later, this low level of production developed into a much more pronounced change accompanying senescence (Fig. 3).

In both genera, the emasculation-induced ethylene production was suppressed under humid conditions, showing a much smaller peak in *Cymbidium* (Fig. 2) and no increase at all in *Phalaenopsis* (Fig. 3).

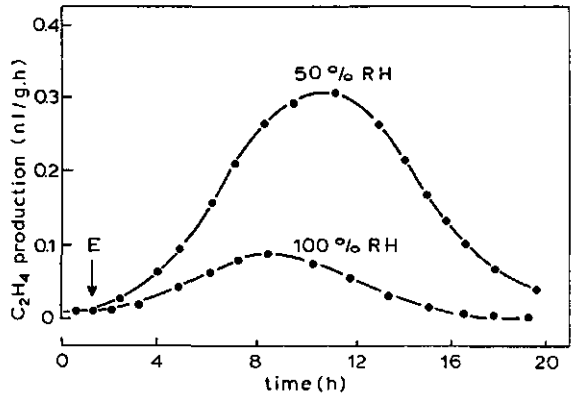

Fig. 2. Changes in ethylene production following emasculation of *Cymbidium* flowers under conditions of low RH (~ 50%) and high RH (~ 100%). Under both conditions, the ethylene production of non-emasculated flowers was low (~ 0.015 nl/g.h) throughout the experiment (data not included). E = time of emasculation.

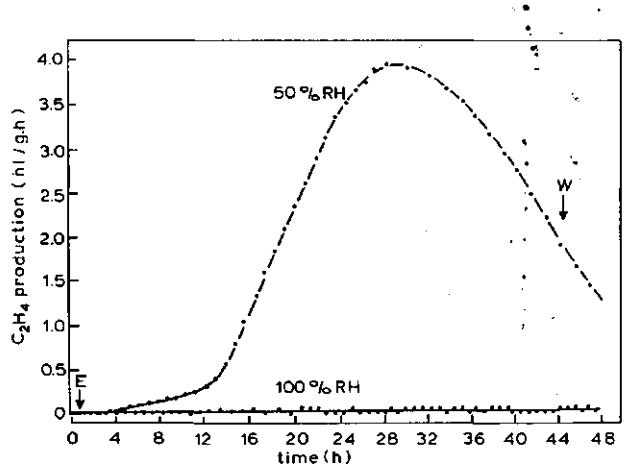

Fig. 3. Pattern of ethylene production following emasculation of *Phalaenopsis* flowers under conditions of low RH (~ 50%) and high RH (~ 100%). Under both conditions the ethylene production of non-emasculated flowers was low (~ 0.02 nl/g.h) throughout the experiment (data not included). E = time of emasculation W = time of wilting.

The effect on ethylene production of transfer of emasculated *Cymbidium* flowers from conditions of high RH to low RH is shown in figure 4. Upon addition of $CaCl_2$ into the cuvette, the humidity dropped from about 100% to about 50% RH within half an hour and remained at that level. Subsequently, the ethylene production showed a pattern similar to the normal emasculation-induced ethylene peak.

The ethylene production of grease-treated *Cymbidium* flowers under conditions of low RH showed only a minor increase comparable to or even less than that of flowers emasculated under high RH (data not shown).

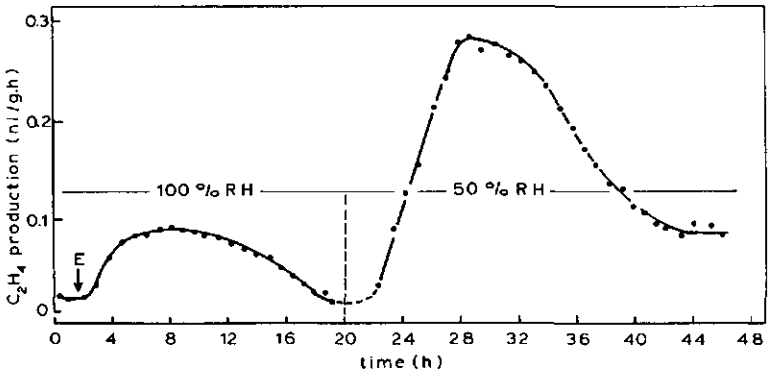

Fig. 4. Effect of delayed desiccation on the ethylene production of emasculated *Cymbidium* flowers.
The ethylene production of non-emasculated flowers was low (~ 0.015 nl/g.h) and showed no significant response to the changing RH (data not included).
E = time of emasculation.

Effect of treatment with AVG and ACC on ethylene production

When emasculated flowers were treated with AVG under conditions of low RH, the emasculation-associated early ethylene production was completely absent. Addition of ACC to a flower that had previously been treated with AVG evoked a rapid increase in ethylene production, comparable to normal emasculation-induced ethylene production (Fig. 5). Similarly, addition of ACC to an emasculated flower placed under conditions of high RH evoked an ethylene surge comparable to the normal response (Fig.5). In these experiments only 20-25% of the applied ACC was recovered as ethylene.

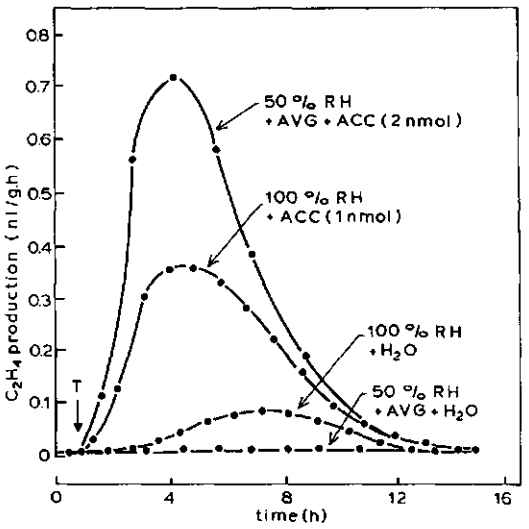

Fig. 5. Effect of ACC and AVG on ethylene production in emasculated *Cymbidium* flowers.
Flowers were placed under conditions of high RH (- 100%) and the rostellum was subsequently treated with water (1.0 μl) or with ACC (1.0 nmol in 1.0 μl). Similarly, flowers treated previously with AVG (50.0 nmol in 10.0 μl onto the rostellum) were placed under conditions of low RH (- 50%) and subsequently treated with water (2.0 μl) or with ACC (2.0 nmol in 2.0 μl).
T = start of treatment.

DISCUSSION

The data presented in this paper provide evidence that desiccation of the rostellar region is the principal factor responsible for post-emasculation phenomena in orchid flowers. In a humid environment, the normal response to emasculation (increased ethylene production, coloration and wilting) is absent and after transfer to conditions of low RH all these effects are restored. This finding is in contrast to earlier suggestions. Mechanical wounding of the rostellum (Arditti and Flick, 1974) and the protecting role of cytokinins present in the pollinia and anther cap (Van Staden, 1979) appear to be of minor importance in post-emasculation phenomena.

Our studies in which the rostellum was treated with AVG (an inhibitor of ACC-synthase) and ACC (the immediate precursor of ethylene) revealed that ACC and ethylene production are an integral part of the emasculation response.

Under humid (non-responsive) conditions, applied ACC is able to evoke a response similar to normal emasculation-induced phenomena and, under conditions of low RH, the emasculation-induced response is inhibited by AVG.

In *Cymbidium*, the effect of AVG on lip coloration could be fully restored by an additional treatment of the rostellum with 2.0 nmol ACC. However, wilting in AVG-treated *Phalaenopsis* flowers could not be restored by the ACC treatment. Similarly, wilting of petals and sepals in AVG-treated *Cymbidium* flowers was also not restored by the ACC treatment (data not shown). An explanation may be that lip coloration in *Cymbidium* can be induced by a relaitvely small amount of ethylene derived from the applied ACC. Wilting, on the contrary, may require a greater amount of ACC and ethylene that has to be synthesized by the tissue itself in response to the applied ACC. As AVG-treated flowers lack this ability, the ACC treatment does not induce wilting in these flowers.

When emasculated flowers which had not been treated with AVG, were placed under humid (non-responsive) conditions, the ACC treatment induced wilting in both species which indicates that, in this case, the relatively small amount of applied ACC was able to initiate the autocatalytic process responsible for wilting.

The data collectively indicate a sequence of events following emasculation starting with desiccation of the rostellar region. This presumably induces the synthesis of ACC which in turn leads to the production of ethylene. Like other stresses, such as mechanical wounding (Yu and Yang, 1980), gravitropic stimulation (E.J. Woltering; unpublished results, 1987), and auxin-induced stress (Yu and Yang, 1979), water stress is known to stimulate ACC-synthase activity (Apelbaum and Yang, 1981). However, the means by which stress leads to increases in enzyme activity is not clear.

Hoekstra and Weges (1986) suggested that in pollinated or stigma-wounded *Petunia* flowers the wounding of the stigma causes the production of elicitors of the type observed after mechanical perturbation or fungal infection. Although, in orchids, the mechanical act of emasculation itself does not induce senescence (Fig. 1), desiccation or merely death of a limited amount of rostellum cells may lead to the release of cell wall fragments (endogenous elicitors) which have been shown to stimulate ACC and ethylene production in other systems (*e.g.* Roby *et al.*, 1985; Tong *et al.*, 1986).

Following emasculation, the initial events are probably restricted to a limited area in the top of the central column, but lip coloration in *Cymbidium* is already visible within 24 h. It is of interest which signal,

evoked by desiccation of the rostellum, may be responsible for this rapid response in a flower organ several centimeters away from the site of desiccation. In carnation, it was demonstrated that there is an increase in ethylene production and ACC content in all the different flower parts following pollination (Nichols *et al.*, 1983). Similarly, ACC levels in *Petunia* corollas increase after wounding of the stigma (Nichols and Frost, 1985). Transport of ACC in pollinated *Petunia* flowers has been suggested by Hoekstra and Weges (1986) and, furthermore, it has been demonstrated that, in carnation, stigma-applied ACC could indeed be transported to the petals (Reid *et al.* 1984).

Coloration of isolated *Cymbidium* lips was shown to be a response entirely regulated by endogenously produced ethylene (Woltering, 1989). It may therefore be suggested that, following emasculation, ACC is transported from the central column to the other flower parts where it is converted to ethylene.

Besides the possible existence of an inter-organ ACC flow it has recently been suggested that in various flowers a second signal, believed to render the tissue more sensitive to ethylene, is involved in pollination-induced senescence (Halevy *et al.*, 1984; Halevy, 1986).

The possible involvement of ACC and other mobile wilting factors in emasculation-induced phenomena in orchid flowers will be the subject of further research.

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Chapter 7

INTERRELATIONSHIP BETWEEN THE DIFFERENT FLOWER PARTS DURING EMASCULATION-INDUCED SENESCENCE IN *CYMBIDIUM* FLOWERS

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SUMMARY

In *Cymbidium* flowers emasculation by removal of the anther cap and the pollinia, led to rapid coloration of the lip and advanced wilting of the petals and sepals. The ethylene production of whole flowers showed an emasculation-induced early peak in ethylene evolution followed some days later by a second increase concomitant with the wilting of the flower. In non-emasculated flowers the ethylene production increased later, simultaneous with coloration of the lip and wilting of the petals and sepals. The contribution of the lip, petals, and sepals to the total amount of ethylene produced was negligible during all stages of senescence.

Parallel to the increase in ethylene production of whole flowers, an increase in 1-aminocyclopropane-1-carboxylic acid (ACC) and malonyl-ACC in the central column and, to a lesser extent, in the ovary was observed. An increase in internal ethylene concentration was also demonstrated and this, in contrast, was apparent in all the different flower parts. The activity of the ethylene-forming enzyme in lips, petals, and sepals showed an increase after emasculation and such an effect could also be induced by treatment of isolated parts with low concentrations of ethylene.

The data indicate that senescence in *Cymbidium* flowers is regulated by the central column and perhaps the ovary and that both ACC and ethylene may play a signalling role in inter-organ communication.

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INTRODUCTION

In orchid flowers senescence can be advanced by removal of the pollinia and the anther cap (emasculation). The initial event in emasculation-induced phenomena was recently shown to be desiccation of the rostellum, an organ of stigmatic origin to which the pollinia are attached (Woltering and Harren, 1989). In *Cymbidium* orchids, emasculation initially evokes a small peak in ethylene production and subsequent red coloration of the labellum (lip). Eventually, emasculated flowers show an earlier appearance of the sudden upsurge in ethylene production that accompanies wilting of the petals and sepals (Goh *et al.*,1985; Woltering *et al.*, 1988).

The visible symptoms of emasculation, *i.e.* coloration and wilting, were shown to be quite similar to the symptoms observed after treatment of the flowers with exogenous ethylene (Arditti *et al.*, 1973). Studies with inhibitors of ethylene biosynthesis revealed that 1-aminocyclopropane-1-carboxylic acid (ACC) and ethylene productions are an integral part of the emasculation response (Woltering and Harren, 1989). In addition, treatment of the flowers with inhibitors of ethylene production or action, were shown to delay emasculation-induced coloration of the lip (Van Staden and Davey, 1980; Goh *et al.*, 1985; Harkema and Struijlaart, 1989). Hence, it may be suggested that the emasculation-induced events in the rostellar region lead to the ethylene-associated phenomena in the other flower parts.

Coloration of isolated *Cymbidium* lips was recently studied in detail (Woltering, 1989). It was shown to be regulated by endogenously produced ethylene and therefore it was argued that, upon emasculation, the lip may show an increased ethylene production or an increased ethylene sensitivity. A similar argument may explain the emasculation-induced wilting in petals and sepals. The question arises which signal links the events in the rostellar region with, for instance, the rapid response in the lip.

The effects of emasculation in *Cymbidium* flowers may be similar or comparable to the effects of pollination. The latter is known to dramatically hasten senescence of, for instance, *Petunia* and carnation flowers and it was suggested that an inter-organ translocation of the immediate precursor of ethylene, ACC, may play a signalling role (Reid *et al.*, 1984; Hoekstra and Weges, 1986). Furthermore, it was suggested that other factors, that are thought to render the tissue more sensitive to ethylene, are translocated in pollinated flowers (Halevy *et al.*, 1984; Halevy, 1986). In fact, two possible candidates, i.e. decanoic acid and octanoic acid were recently isolated from

eluates of pollinated *Petunia* styles (Whitehead and Halevy, 1989).

For a better understanding of the mechanism through which emasculation leads to advanced senescence, the changes in ethylene production, internal ethylene concentrations, the contents of ACC and malonyl-ACC (MACC), and the activity of the ethylene-forming enzyme (EFE) were studied in detail in different parts of the *Cymbidium* flower.

MATERIALS AND METHODS

Plant material and vase life studies

Flower spikes were obtained from a commercial grower and transported dry to the laboratory. The experiments were carried out with individual flowers cut from the middle region of the spikes. After excision, the flowers were placed in water under controlled environmental conditions of 12 h white light (15 $\mu mol/m^2.s$) and 12 h darkness; 60% relative humidity (RH) and 20°C throughout.

Unless otherwise indicated, *Cymbidium* cv. Jacobi was used in the experiments. In all cases, a comparison was made between flowers from which the anther cap and the pollinia had been removed (emasculated) and non-emasculated (control) flowers.

The development of senescence symptoms was visually evaluated each day. The average number of days to lip coloration (color score ≥ 3) and wilting was calculated. Lip coloration was classified on a scale from 0 (original color) to 4 (dark-red coloration) with a discrimination of about 0.5 using color prints (Woltering, 1989).

When the flowers were divided into the different parts to be used for further analysis, the following definitions were used:

- upper part of column = upper third of the central column always without the anther cap and the pollinia, but containing the stigma and the rostellum
- lower part of column = lower two thirds of the central column, excised just above the ovary
- lip = excised labellum (= modified petal)
- petals = the two remaining petals in the inner whorl
- sepals = the three sepals (with the appearance of petals) in the outer whorl.

Measurement of ethylene production

Ethylene production of whole flowers was measured regularly by enclosing two flowers in a 270 ml glass container. Before the containers were closed, they were flushed with compressed air, which was first led through a column filled with Ethysorb (aluminum oxide coated with $KMnO_4$, Stay Fresh Ltd., London, U.K.) to remove hydrocarbons. After 1 to 3 h incubation (depending on the stage of senescence) the ethylene concentration in the headspace was measured by gas chromatography (GC). The gas chromatograph (Intersmat, Pavillion du Bois, France) was equipped with a stainless steel column filled with Alumina-GC (Chrompack, Middelburg, The Netherlands) and a flame ionization detector. The minimum detection limit for ethylene was approximately 2 nl/l. During the incubation period, the ethylene concentration generally did not exceed a level of 50 nl/l; the carbon dioxide concentration never exceeded 0.5% (v/v). It had previously been found in a separate experiment that these concentrations of ethylene and carbon dioxide did not have any effect on the rate of ethylene production.

The ethylene production during the first day after emasculation was measured using a flow-through system in line with a sensitive laser photoacoustic detector. The minimum detection limit for ethylene was approximately 0.03 nl/l (Woltering *et al.*, 1988).

The ethylene production of excised flower parts was measured by enclosing three of each in a 30 ml glass vial for approximately 1 to 2 h. In this way, the interference with wound-induced ethylene, which was observed in excised columns after 2 h of incubation, was avoided. Prior to closure, the vials were flushed for several min with hydrocarbon-free air. Ethylene in the headspace was analysed by GC.

Analysis of ACC and MACC

At each collection time, 10 to 12 flowers were frozen in liquid nitrogen and dried under vacuum. The dried flowers were divided into their different parts, ground in a mini ball-mill (Retsch MM2, West Germany) and a sample of 0.2 g was extracted with 4 ml methanol (80%). After centrifugation for 30 min at 30,000 g an aliquot of 2 ml of the supernatant was dried in vacuum at 45°C and the residue re-dissolved in 2 ml distilled water.

ACC was analysed in the aqueous extract according to the method of Lizada and Yang (1979) using internal standardization with authentic ACC (Sigma, St. Louis, USA). ACC was also determined after treatment of the extract with an

equal volume of 6 mol/l HCl (2 h at 100°C) and neutralization with NaOH. The difference between the data obtained with the two procedures was taken as the amount of MACC.

Internal ethylene concentrations

At each sample date, the internal ethylene concentration of lips, petals, sepals, columns, and ovaries was analysed by vacuum extraction of the internal gas phase according to the procedure described by Beyer and Morgan (1970). Parts excised from 12 flowers were used for each measurement. The parts were subjected to a vacuum of 20 mbar pressure for approximately 2 to 3 min and thereafter, 0.3 ml of the extracted gas was analysed for ethylene.

EFE-activity

For determination of the *in vivo* EFE-activity the lips, petals, and sepals were excised and placed in 30 ml vials with their cut base in 2 ml, 10 mmol/l ACC-solution. Lips were incubated individually, all petals and sepals excised from one flower were incubated together. Two hours after start of the incubation, the vials were flushed with hydrocarbon-free air and closed with a screw cap containing a septum. Approximately 1 h later, a sample of the headspace was analysed for ethylene.

Treatment of isolated lips with ACC and ethylene

To establish the relation between the ethylene production and the internal ethylene concentration, lips were excised and placed with their cut base in different concentrations of ACC (0, 0.005, 0.025, 0.05, 0.1, 0.2, 0.5, and 1.0 mmol/l). After approximately 24 h the lips were enclosed in 270 ml glass containers (12 lips each) for about 0.5 h. Thereafter the ethylene concentration in the headspace was measured by GC and the lips were immediately assayed for internal ethylene.

Isolated lips, placed with their cut base in water were treated with ethylene (0, 0.1, and 0.2 µl/l) by enclosing them in 70 l stainless steel chambers at 20 ± 1°C in darkness. During the treatments, the carbon dioxide concentration was kept low (< 0.05%; v/v) and the oxygen concentration was maintained at ambient level (Woltering and Sterling, 1986).

RESULTS

Lip coloration and wilting

Emasculation-induced lip coloration and wilting were determined in different cultivars. Within approximately one day after emasculation, the lips showed a slightly pink coloration which developed into dark-red within the following days. Several days later, the petals and sepals showed the first signs of senescence *i.e.*, discoloration, inrolling and shrivelling (Table 1). In non-emasculated flowers, lip coloration and wilting of petals and sepals manifested themselves after a longer time span and the two phenomena appeared much closer to each other in time.

Time to coloration and wilting varied between the different cultivars and also between the different experiments. However, despite these differences, the response of all the tested cultivars was essentially the same.

Table 1. Effect of emasculation on the average number of days to lip coloration (color score ≥ 3) and wilting in different *Cymbidium* cultivars (n=10).

Cultivar	Emasculation	Time to lip coloration (days)	Time to wilting (days)
Jacobi	-	8	12
	+	1.5	9
Mary Pinchess Del Rey	-	6	9
	+	1	3
Showgirl Malibu	-	8	10
	+	2	9
King Arthur	-	11	15
	+	1.5	12
Silvia Miller	-	10	18
	+	1.5	12
Ivy Fung Sultan	-	14	18
	+	1.5	12

Ethylene production

The pattern of ethylene production in whole flowers is shown in figure 1. In emasculated flowers, the ethylene production showed an increase within a few h after treatment; the production peaked about 10 h later and then decreased to a level just slightly above the control. Several days later the

production again showed an increase preceding the visible wilting of the flowers. In non-emasculated flowers the ethylene production remained low during the first six days of vase life and thereafter an increase was observed prior to coloration of the lip and wilting of the flower.

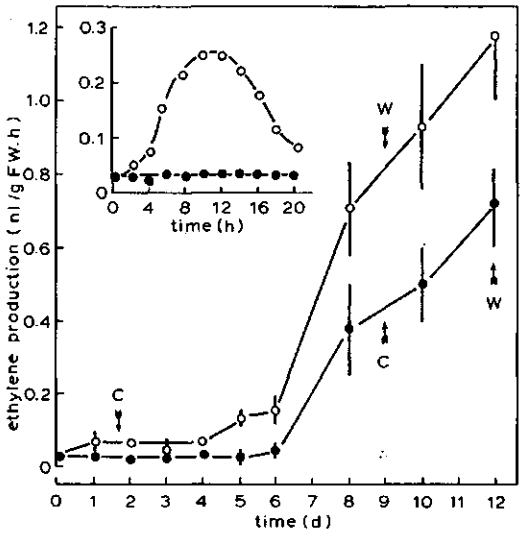

Fig. 1. Pattern of ethylene production in emasculated (o) and non-emasculated (•) *Cymbidium* 'Jacobi' flowers.
The flowers were emasculated at t = 0. Values are means of three containers with 2 flowers each; vertical bars represent 2 x s.e., where s.e. bars were larger than symbols; C = time of coloration (color score $\geq$ 3); W = time of wilting.
The insert shows the ethylene production of individual flowers during the first 20 h of vase life measured by laser photoacoustic detection.

At three different stages of senescence the ethylene production of excised flower parts was measured, *i.e.* during the first day, during the lag-phase, and during the upsurge in ethylene production. Both in emasculated and non-emasculated flowers, regardless of the stage of senescence, the production of the isolated lips, petals, and sepals was very low. In contrast, high rates were observed in excised columns (Table 2). This indicates that the perianth may have very little capacity to synthesize ACC or to convert ACC to ethylene.

Table 2. Ethylene production of different flower parts excised at three different stages of senescence.
A = during the first day (~ 0.5 d)
B = during the lag-phase (- 3 d)
C = during the terminal phase (- 10 d)
Columns and ovaries were measured while united; 2 petals and 3 sepals, being one unit, were measured while separated from each other.
Values are means ± standard error of two vials with 3 units each.

Stage of senescence	Flower part	Ethylene production (nl/unit.h)	
		Emasculated	Non-emasculated
A	Column + ovary	2.6 ± 0.21	0.05 ± 0
	Petals + sepals	0.06 ± 0.014	0.02 ± 0
	Lip	0.02 ± 0	0.02 ± 0
B	Column + ovary	0.4 ± 0.04	0.1 ± 0.02
	Petals + sepals	0.05 ± 0.014	0.02 ± 0.06
	Lip	0.04 ± 0.008	0.02 ± 0
C	Column + ovary	6.2 ± 0.14	2.9 ± 0.20
	Petals + sepals	0.17 ± 0.04	0.13 ± 0
	Lip	0.05 ± 0.007	0.04 ± 0.007

Internal ethylene concentrations in different flower parts

In emasculated flowers, a significant increase in ethylene concentrations in all the flower parts was observed during the first day after emasculation (Fig.2). Especially in the central column the concentration dramatically increased (up to 4 μl/l). On the 4th day of the vase life a second increase in all the different flower parts appeared simultaneously, reaching values of up to 2 μl/l in lips, petals, and sepals; 5 μl/l in the ovaries, and 15 μl/l in the columns. In non-emasculated flowers, a simultaneous increase in internal concentrations in all the flower parts was observed on the 5th day of vase life. In these flowers the accumulation of ethylene occurred at a slower rate than in emasculated flowers (Fig. 2).

There was a good correlation between the internal ethylene concentrations in different flower parts and the ethylene production of the whole flowers (Fig. 3). In a separate experiment, the relation between ACC-induced ethylene production and the internal ethylene concentration in isolated lips was established. This also revealed a good linear relationship (Fig. 3, insert).

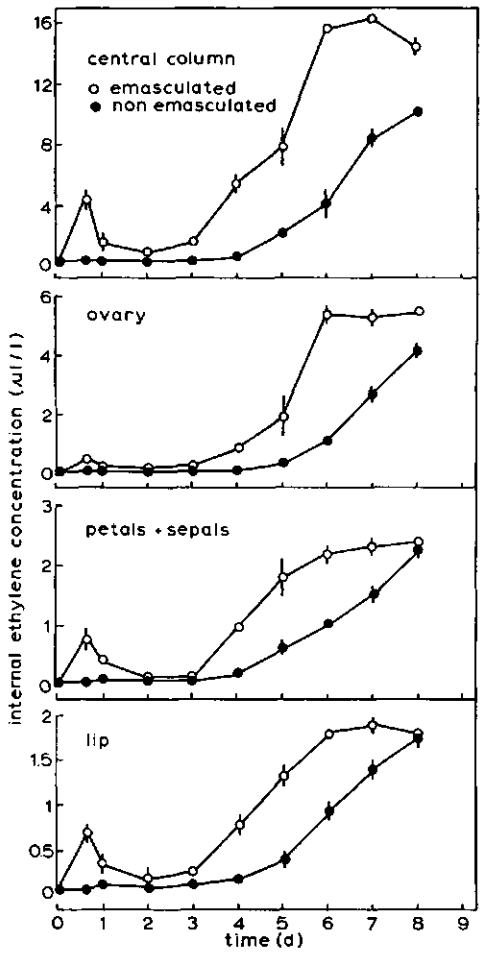

Fig. 2. Time course of the internal ethylene concentration (different scales) in different parts of emasculated (o) and non-emasculated (•) *Cymbidium* 'Jacobi' flowers.
The flowers were emasculated at t = 0. Values are means of two measurements, each performed with parts from 12 flowers; vertical bars represent 2 x s.e., where s.e. bars were larger than symbols.

ACC and MACC in different flower parts

During the course of vase life the contents of ACC and MACC were measured in different flower parts: upper part of the column, lower part of the column, ovary, petals + sepals, and lips. Both in emasculated and in non-emasculated flowers, only negligible changes in ACC and MACC were found in lips, petals and sepals during the first 10 days of vase life. Thereafter, a

slight increase in the ACC content of the petals and sepals of both emasculated and non-emasculated flowers was observed (data not shown). In emasculated flowers, there was a pronounced increase in both ACC and MACC in the upper part of the central column during the first day of vase life.. Thereafter, the ACC content showed a gradual decrease while the increase in MACC temporarily ceased (Fig. 4). In the lower part of the column no significant change in ACC or MACC was observed until the fourth day after emasculation; the increase in ACC in the lower part of the column appeared simultaneously with an increase in the ovary (Fig. 4).

In non-emasculated flowers a simultaneous increase in ACC and, to a lesser extent, in MACC was observed towards the end of the vase life in both portions of the central column and in the ovary; the MACC content in flower parts of non-emasculated flowers gradually increased during vase life (Fig. 4). The changes in the levels of ACC and MACC in both emasculated and non-emasculated flowers showed a close correlation with the increase in ethylene production of whole flowers (compare Figs. 1 and 4).

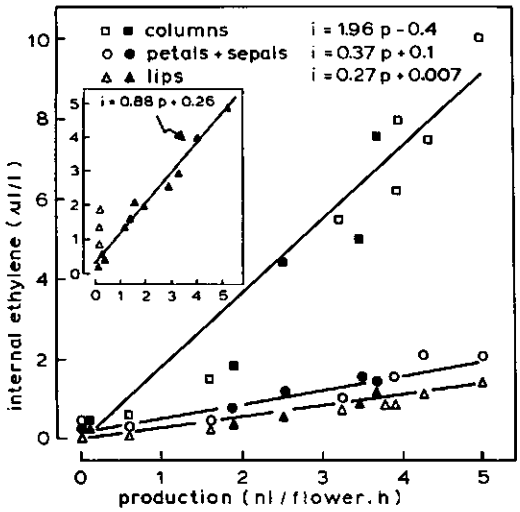

Fig. 3. Relation between the internal ethylene concentration in different organs (i) and the ethylene production of whole flowers (p).
Data are from two separate experiments. Ethylene production was measured in different flowers than those which were used for determination of the internal concentration. Open symbols represent data from emasculated flowers; solid symbols from non-emasculated flowers.
Insert: Relation between internal ethylene (i) and production (p) in lips treated with different concentrations of ACC (▲) and in lips excised from emasculated flowers (△).

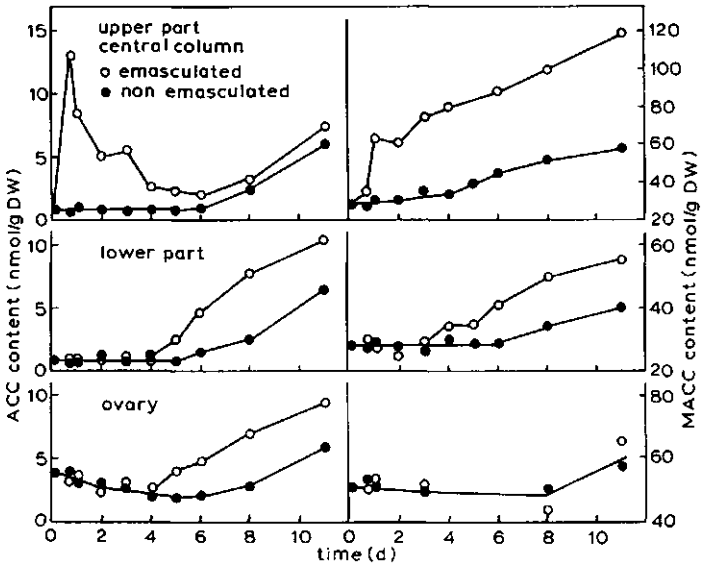

Fig. 4. Changes in the ACC content (left) and the MACC content (right) of different parts of emasculated (o) and non-emasculated (•) *Cymbidium* 'Jacobi' flowers as a function of time.
The flowers were emasculated at t = 0. Values are means of 3 to 6 analyses in an extract derived from tissue of 12 flowers. Standard errors generally were less than 10% of the mean.

EFE-activity in different flower parts

In emasculated flowers there was a pronounced increase in EFE-activity in the lips within one day after emasculation (Fig. 5). This activity gradually increased further thereafter. In control flowers the EFE-activity in the lip started to rise at a much later time (Fig. 5). In petals and sepals, emasculation initially induced a small but statistically significant rise in EFE-activity which developed some days later into a much more pronounced increase. In petals and sepals excised from non-emasculated flowers, the increase in EFE-activity started later (Fig. 5).

When lips were excised from emasculated flowers and were placed with their base in water, the EFE-activity of the isolated lips showed a decline (Fig. 6). In lips excised from control flowers at a time when the EFE-activity was still very low, the normal increase in EFE-activity remained absent. When lips were isolated from fresh flowers and exposed to ethylene, there was already a marked increase in EFE-activity at low concentrations (Fig. 6, insert).

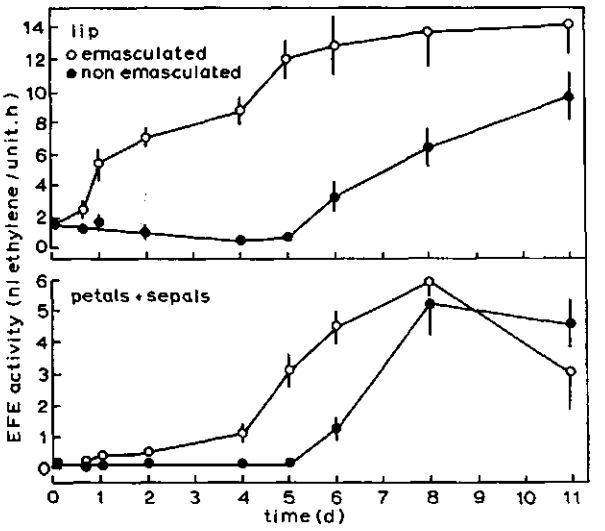

Fig. 5. Time course of the EFE-activity in different parts of emasculated (o) and non-emasculated (•) *Cymbidium* 'Jacobi' flowers.
The flowers were emasculated at t = 0. Lips were excised and incubated individually in 30 ml vials with their cut base in a solution of 10 mmol/l ACC; petals and sepals excised from one flower were incubated together. Values are means of 5 measurements; vertical bars represent 2 x s.e., where s.e. bars were larger than symbols.

DISCUSSION

When *Cymbidium* flowers are emasculated they show advanced senescence as determined by an earlier coloration of the lip and wilting of the petals and sepals. In all tested cultivars, severe coloration of the lip was apparent within two days after emasculation and all cultivars showed advanced wilting. The biochemical events leading to senescence in cv. Jacobi as discussed below may therefore also be representative for other cultivars.

Coloration and wilting in emasculated flowers coincided with the changes in ethylene production of the flower. Coloration appeared directly after the first increase in ethylene production, wilting during the second rise in ethylene evolution. In non-emasculated flowers, coloration occurred during the early phase of the rise in ethylene production and was followed only a few days later by wilting of the petals and sepals (Fig. 1). Ethylene production and senescence symptoms thus seem to be connected.

In *Petunia* it was found that the early pollination-induced ethylene production was exclusively derived from the gynoecium, whereas the second increase was mainly derived from the corolla. Therefore it was argued that the early ethylene production did not control corolla senescence but that corolla ethylene during the second increase was responsible (Hoekstra and Weges, 1986). In all stages of senescence in *Cymbidium* flowers, the excised "central column + ovary" part of the flower produced significant amounts of ethylene. However, throughout senescence the excised lip, petals, and sepals only contributed to a minor extent to the total production (~ 2%; Table 2). As these latter organs show the visible signs of senescence (coloration and wilting) it may be argued that, unlike the situation in *Petunia*, local ethylene synthesis is not the cause of perianth senescence in *Cymbidium*.

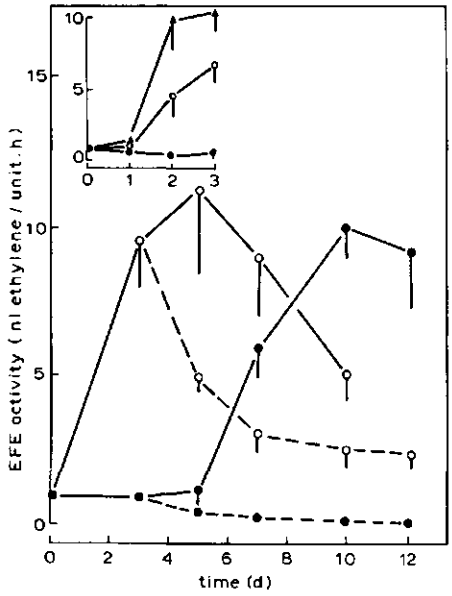

Fig. 6. EFE-activity in lips still attached to the flower (continuous lines) or excised at t = 3 d (dotted lines) from emasculated (o) and non-emasculated (●) *Cymbidium* 'Jacobi' flowers.
After excision the lips were placed with their cut base in water.
Insert: Effects of continuous treatment with 0 (●), 0.1 (o) and 0.2 µl/l (▲) ethylene on isolated lips placed in water.
Values are means of 5 measurements; vertical bars represent 1 x s.e., where s.e. bars were larger than symbols.

Immediately after emasculation, an increase in both ACC and MACC was only observed in the upper portion of the central column. This indicates that at this early stage of senescence the rostellar region is the sole site of ACC synthesis, probably being induced by localized desiccation (Woltering and Harren, 1989). At a later stage, also the lower part of the column and the ovary may participate in ACC synthesis (Fig. 4). In non-emasculated flowers there is a simultaneous increase in both ACC and MACC in the upper and lower parts of the central column and in the ovary during the later stage of senescence. In contrast, both in emasculated and in non-emasculated flowers, the ACC and MACC contents remained at a low level in the entire perianth throughout the vase life.

These data collectively indicate that the column and the ovary are the sites of ACC and ethylene synthesis in all stages of senescence. This is contrary to the situation in *Petunia* and carnation where increases in ACC and ethylene productions in the corolla were evident during senescence (Nichols *et al.*, 1983; Nichols and Frost, 1985). The perianth of *Cymbidium* seems to have very little capacity to synthesize ACC, probably as a result of a low ACC-synthase activity.

In contrast to the activity of ACC-synthase, the activity of the ethylene-forming enzyme is rapidly induced by emasculation in lips and thereupon also in petals and sepals (Fig. 5). In non-emasculated flowers, the EFE-activity in the perianth started to rise much later. After removal of the lip from an emasculated flower, the EFE-activity showed a gradual decline. Similarly, the increase in EFE-activity in lips from non-emasculated flowers was prevented when this organ was excised prior to the induction of EFE-activity (fig. 6). These data indicate that some factor originating from the remaining portion of the flower induces EFE-activity in the lip. From this experiment, a half-life of about 2.5 d for the ethylene-forming enzyme could be calculated. This is quite similar to the half-life of ethylene-induced EFE-activity in preclimacteric fruits (Liu *et al.*, 1985).

In isolated lips low concentrations of ethylene already markedly induced EFE-activity (Fig. 6, insert). This observation is in line with results in preclimacteric cantaloupe and apple fruits and in carnation petals where ethylene was shown to induce this activity (Manning, 1985; Liu *et al.*, 1985; Bufler, 1986).

Although the central column and the ovary were found to be the site of ACC and ethylene synthesis throughout the vase life, an early emasculation-induced increase in internal ethylene concentration was found in all the

different flower parts (Fig. 2). Later during senescence, again a simultaneous increase in internal ethylene was found in all the flower parts. Taking into consideration that during separation of the flower parts some ethylene may already diffuse out and that adhesive air may dilute the final concentration, the internal concentrations may have been even higher. The increase of EFE-activity in lips and petals may therefore be a result of the accumulation of ethylene.

The changes in internal concentration showed a close correlation with the changes in ethylene production of whole flowers (Fig. 3). Similarly, the accumulation of ethylene in isolated lips placed in different concentrations of ACC was closely correlated with their ethylene production (Fig. 3, insert). This indicates that, in *Cymbidium* flowers, small changes in ethylene production significantly affect the internal ethylene concentrations and will therefore have physiological effects. In addition, it shows that the internal ethylene concentrations observed in lips excised from emasculated flowers may not be related to their ethylene production (Fig. 3, insert).

To explain these conflicting results it is suggested that ethylene produced in the central column, where it reached concentrations of up to 15 μl/l, is translocated to the other flower parts where it induces coloration and wilting. The ethylene concentrations which were observed in lips after emasculation were indeed shown to be high enough to induce coloration in isolated lips of *Cymbidium* cv. King Arthur when applied exogenously (Woltering, 1989). This was also found to be true in cv. Jacobi (data not shown). These concentrations are also sufficient to induce the observed increase in EFE-activity (Fig. 6, insert). Inter-organ translocation of ethylene has previously been suggested to play a role in the response of tomato plants to waterlogging and it has been shown that radio-labeled ethylene can indeed be transported from the roots to the shoot (Jackson and Campbell, 1975).

Another explanation for the discrepancy between the measured ethylene production and the levels of internal ethylene is that ACC, which is produced in the central column, may be translocated to the other flower parts where it is immediately converted to ethylene. In this case the ethylene production of an isolated organ may rapidly decrease after excision due to the lack of substrate. The internal concentration, on the contrary, may stay high for some time due to poor diffusion through the thick waxy cuticle. Transport of endogenous ACC was suggested to occur in pollinated carnation and *Petunia* flowers (Nichols *et al.*, 1983; Hoekstra and Weges, 1986) and it was shown that, in

carnation, stigma-applied ACC was rapidly translocated to the petals (Reid *et al.*, 1984). In fact, we recently demonstrated that, in *Cymbidium*, endogenous ACC is indeed translocated from the central column to the perianth (Woltering, 1990).

Besides the translocation of ACC, it has been suggested that other factors, that are thought to render the tissue more sensitive to ethylene, may be translocated in pollinated flowers (Halevy *et al.*, 1984; Whitehead and Halevy, 1989). Based on the data of local ACC and ethylene productions in lips, petals, and sepals during senescence of *Cymbidium* flowers one might conclude that the ethylene effects observed in the perianth are due to an increased sensitivity to the low, basal levels of ethylene. However, measurements of the internal concentrations reveal that the accumulation of ethylene rather than an increased sensitivity is the cause of the observed senescence symptoms.

This study provides a picture of the regulation of petal senescence in *Cymbidium* flowers. During senescence of non-emasculated flowers, increases in ethylene and ACC in the central column and, to a lesser extent, in the ovary lead to the accumulation of ethylene in the lip, petals, and sepals. This increase in internal ethylene concentration in the perianth, which may be the result of the translocation of ACC, ethylene, or a combination of both, induces coloration of the lip and wilting of the petals and sepals. In emasculated flowers, an early increase in ACC in the top of the central column leads to a transient increase in internal ethylene in the perianth. This increase induces rapid coloration of the lip and an increase in EFE-activity. It is suggested that the enhanced EFE-activity may cause an increased production and, in particular, influx of ACC in the perianth, thereby stimulating ethylene production that causes accelerated senescence in comparison to non-emasculated flowers.

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CHAPTER 8

INTER-ORGAN TRANSLOCATION OF 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID AND ETHYLENE COORDINATES SENESCENCE IN EMASCULATED *CYMBIDIUM* FLOWERS

Ernst J. Woltering

SUMMARY

In *Cymbidium* flowers, emasculation by removal of the pollinia and the anther cap leads within 24 h to red coloration of the labellum (lip), a phenomenon that has been ascribed to the action of ethylene. When a small incision in the base of the lip is made prior to emasculation, or when the lip is excised and placed in water within 10 to 15 h after emasculation, coloration is considerably delayed.

Measurements of ethylene production of excised flower parts, isolated at different times after emasculation, showed an increase only in the central column; the other flower parts, including the lip, did not show a measurable change. In contrast, *in situ* measurements of the ethylene production of the central column and the remaining portion revealed a simultaneous increase in all the flower parts following emasculation. Similarly, application of radio-labeled 1-aminocyclopropane-1-carboxylic acid (ACC) to the top of the central column *in situ* leads to the production of radio-labeled ethylene by all the flower parts.

Treatment of the central column *in situ* with ethylene or ethephon did not stimulate ACC production but did stimulate lip coloration and this was accompanied by an increased internal ethylene concentration in the lip.

The data show that ACC as well as ethylene are translocated from the site of production to all the other flower parts, thereby providing a mechanism for coordination of the senescence process.

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INTRODUCTION

Inter-organ translocation of 1-aminocyclopropane-1-carboxylic acid (ACC) was first described by Bradford and Yang (1980) for the root-to-shoot transport in tomato. In this species, this xylem transport of ACC was also suggested to be involved in the pathogenic symptom expression upon root-knot nematode infection (Glazer *et al.*, 1984). Amrhein *et al.* (1982) demonstrated that, also in tomato plants, application of labeled ACC to a single leaflet leads to the accumulation of radioactivity in other leaves and in the roots indicating that ACC may also be transported through the phloem.

ACC may also be translocated between the different organs of a flower. Nichols *et al.* (1983) observed increased ACC levels in all the different flower parts after pollination of carnation and hypothesized that this may at least partly be due to translocated ACC. Stigma wounding in *Petunia* causes an increase in ACC, first in the stigma and then in the corolla (Nichols and Frost, 1985) suggesting ACC translocation. Although direct proof was lacking, Hoekstra and Weges (1986) also argued that ACC may be the transported wilting stimulus in pollinated *Petunia* flowers. In isolated carnation petals, ACC was found to be synthesized in the lower portion and was thought to be transported to the upper portion during the course of senescence (Mor *et al.*, 1985). Reid *et al.* (1984) provided direct proof for the translocation of ACC in carnation flowers by detecting the production of radio-labeled ethylene by the petals from stigma-applied radio-labeled ACC. The high amount of ACC used in this study, however, made it difficult to judge the significance of this finding for the natural situation.

It has been suggested that mobile wilting factors other than ACC may also be translocated in pollinated flowers (Halevy, 1986). Eluates from pollinated *Petunia* styles have been shown to posess wilt-inducing properties while these eluates did not contain any detectable ACC or ethylene (Gilissen and Hoekstra, 1984). Only recently, in fact, two possible candidates i.e. decanoic acid and octanoic acid, were isolated from eluates of pollinated *Petunia* styles (Whitehead and Halevy, 1989). In *Cyclamen* flowers, pollination-induced corolla abscission was ascribed to the action of a pollination-induced ethylene-sensitivity factor as abscission in pollinated flowers could be prevented by silver thiosulphate whereas it could not be induced by ethylene or ACC in non-pollinated flowers (Halevy *et al.*, 1984). Similarly, it was suggested that such a mobile ethylene-sensitivity factor was produced in the lower portion of the carnation petal (Mor *et al.*, 1985).

In *Cymbidium* flowers, senescence can be advanced by emasculation, i.e. removal of the pollinia and the anther cap. Within a few h after emasculation, the ethylene production of the flower shows a small peak that lasts about 24 h (Woltering *et al.*, 1988). This emasculation-induced ethylene production was found to result from desiccation of the rostellum, originally covered by the anther cap and the pollinia, and was associated with an increased synthesis of ACC (Woltering and Harren, 1989). Approximately 24 h after emasculation, the labellum (lip), an organ several cm away from the site of desiccation, shows a slightly pink coloration which develops into dark-red within the following days (Woltering and Harren, 1989). Some days later the perianth shows signs of wilting. Coloration of the lip, either induced by emasculation or by natural ageing may be regarded as the first visible sign of senescence in these flowers.

Coloration of isolated *Cymbidium* lips can be induced by exogenous ethylene or ACC and therefore it was argued that emasculation-induced lip coloration may also involve the action of ethylene, either through translocation of ethylene or ACC or through translocation of an ethylene-sensitivity factor from the site of desiccation to the lip (Woltering, 1989). The emasculated *Cymbidium* flower was therefore chosen as a model to investigate the possible role of ethylene, ACC, and other senescence or wilting factors in inter-organ communication during senescence of flowers.

MATERIALS AND METHODS

Chemicals

Aminoethoxyvinylglycine (AVG) and [^{12}C] ACC were purchased from Sigma (St. Louis, USA); 2,5-norbornadiene (NBD) from Aldrich-Chemie (Steinheim, West Germany); [2,3-^{14}C] ACC from Commissariat a l'Energie Atomique (Gif-sur-Yvette, France) and Ethrel (480 g/l ethephon) from Luxan (Elst, The Netherlands). The radio-labeled ACC solution was supplied in HCl and was buffered to pH 7 with a phosphate buffer solution from Merck (Darmstadt, West-Germany) prior to use. A mixture of ethylene in air was made by combining a known amount of ethylene with compressed air.

Plant material and vase life studies

Cymbidium flower spikes were obtained from a commercial grower and transported dry to the laboratory. The experiments were carried out with individual flowers, cut from the middle region of the spike. Generally, after excision, the flowers were placed in water under controlled environmental conditions of 12 h white light from fluorescent tubes (15 μmol/m^2.s), 12 h darkness at 20°C and 60% relative humidity. Most experiments were performed with cv. Jacobi; in addition, cv. King Arthur and cv. Ivy Fung Sultan were also used in some experiments.

The incidence of lip coloration was visually evaluated each day. Lip coloration was classified on a scale from 0 to 4 using color prints as follows: 0=original color; 1=slightly pink coloration; 2=pink coloration; 3=red coloration and 4=dark-red coloration (Woltering, 1989). Unless otherwise indicated, time to coloration represents the average number of days to red coloration of the lip (color score $\geq$ 3).

Localization and blockage of vessels in the lip

To study the distribution of vessels in the lip, excised lips were placed for a few h with their cut base in an aqueous solution of acid fuchsin. Due to this procedure, the vascular bundles became visible as red stripes. From this experiment, a rough estimation could be made where to make incisions in order to block a certain amount of vessels.

After the incisions were made *in situ*, a small piece of PVC-film was inserted to ensure that no contact between the two parts of the lip could occur.

Treatment of the central column with chemicals

Treatment of the column with AVG was done immediately after emasculation. An aqueous solution (2 to 10 μl depending on the experiment) containing a known amount of AVG was pipeted onto the rostellum and allowed to dry. When the rostellum was also treated with ACC or ethephon, a known amount of these chemicals (aqueous solution) was pipeted onto the rostellum approximately 20 h after the AVG treatment.

Treatments with ethylene, NBD, or a combination of both were carried out by flushing the gases with a flow rate of 0.6 l/h through a 7 mm wide tube, that was placed over the upper half of the central column. NBD vapor of approximately 4000 μl/l was made by flushing compressed air over liquid NBD heated to about 30°C. To prevent condensation, the manifolds and tubing were

also heated. The concentration was calculated by weighing of the NBD solution at intervals. To prevent diffusion of ethylene or NBD from the outlet of the tube to the lip or petals, a ventilator was placed in front of the flowers.

Treatment of the lips with ethylene

Lips, while attached to the flower, were treated for 20 h with ethylene by enclosing them in 15 mm wide tubes that were flushed with ethylene as described above. In this case, a comparison was made between the effect of ethylene on coloration of lips in non-emasculated flowers and in flowers that were emasculated and immediately treated with AVG in a manner as described above.

Alternatively, lips excised from non-emasculated flowers and from flowers that were emasculated and treated with AVG at ca. 15 h prior to excision, were placed in water and treated for 20 h with different concentrations of ethylene. The ethylene treatments were carried out by placing the isolated lips in 70 L stainless steel chambers in which a known amount of ethylene was injected. During the treatments, the carbon dioxide concentration was kept low (< 0.05%; v/v) and the oxygen concentration was maintained at ambient level (Woltering and Sterling, 1986). After the ethylene treatment the development of red coloration was evaluated.

Ethylene production of isolated flower parts

At different times, flowers were dissected and the ethylene production of the different flower parts was measured by enclosing them in 30 ml glass vials (3 parts per vial), allowing ethylene to accumulate. Prior to closure, the vials were flushed with ethylene-free air. Isolated columns and ovaries showed a wound-induced increase in ethylene production starting 1.5 to 2 h after incubation and, therefore, ethylene measurements were done within one h. The isolated lips, petals, and sepals did not show a measurable wound response and were incubated for 3 to 4 h. When isolated lips were treated with ACC they were regularly incubated for about 2 h in 270 ml glass containers (10 lips each).

Samples of the headspace were analysed by gas chromatography (GC) for ethylene. The GC was equipped with a stainless steel column (2.5 m; inner diameter 4 mm) filled with Alumina-GC (Chrompack, Middelburg,The Netherlands) and a flame ionization detector. The minimum detection limit for ethylene was ca. 2 nl/l.

Determination of internal ethylene and ACC concentrations

Internal ethylene concentrations were analysed using the method described by Beyer and Morgan (1970). For each measurement ten lips were exposed for approximately 2 to 3 min to 20 mbar pressure. Thereafter, a 0.3 ml sample from the extracted gas was analysed by GC.

ACC in the central column was estimated approximately 20 h after emasculation or treatment with ethylene. The tissue was frozen in liquid nitrogen, dried in vacuo and extracted with 80% methanol. ACC was analysed by the method of Lizada and Yang (1979) with internal standardization.

***In situ* measurement of ethylene production**

Ethylene produced by the column and the remaining portion of the flower was determined using a flow-through system in line with a laser photoacoustic detector. The minimum detection limit for ethylene was 0.03 nl/l (Woltering *et al.*, 1988). Inside the cuvette the air was directed into two streams (ratio 1:1), one leaving the cuvette via the normal outlet, the other directed through a small tube that was placed over the central column. In this way, the *in situ* ethylene production of the column and the remaining portion could be calculated. The data were corrected for back diffusion (ca. 5%) that was tested by inserting a small amount of ethephon into the column compartment.

Similarly, the production of ethylene in the column and the remaining portion of AVG-treated flowers was measured after addition of 2 nmol ACC upon the rostellum.

Experiments with labeled ACC

The experimental setup described above was also used for determination of the translocation of [2,3-^{14}C] ACC. A small amount (5 nmol) with a specific activity of 3 x 10^6 Bq/μmol was applied to the rostellum of a flower previously treated with AVG. In this case, the air leaving the cuvette was directed through a solution of 0.25 mol/l mercuric perchlorate (each line two vials in series with 10 ml each) to trap the ethylene. After approximately 20 h of incubation, the radioactivity in the mercuric perchlorate solutions was determined with a Packard Tricarb scintillation spectrometer. The flower was separated into different parts which were immediately ground in liquid nitrogen for determination of the radioactivity in methanolic extracts. In addition, a sample of the extract from the top of the central column was run on cellulose thin layer chromatography (TLC) (Merck, Darmstadt, West-Germany;

solvent n-propanol-ammonia/7:3). Radioactivity on the plates was determined with Berthold TLC-scanning equipment.

All experiments were repeated at least once, representative data are shown.

RESULTS

Translocation of a wilting factor

When lips were excised and placed in water within 10 h after emasculation, coloration was completely prevented in the cultivars under study. However, lips isolated at approximately 20 h after emasculation, at a time they did not yet show any visible sign of coloration, generally developed coloration comparable to lips attached to the emasculated flower (Table 1).

Table 1. Coloration of lips isolated at different times after emasculation, for three different *Cymbidium* cultivars (n=10).

Treatment	Time to coloration (d)		
	cv. Ivy Fung Sultan	cv. Jacobi	cv. King Arthur
Not isolated	2	1.5	1.5
Isolated at t = 0 h	11	20	25
at t = 5 h	10	21	28
at t = 10 h	10	19	24
at t = 15 h	6	4	17
at t = 20 h	3	2	2
at t = 25 h	2	1.5	2
at t = 30 h	2	1.5	1.5

To block the presumed translocation of a wilting factor from the column to the lip, small incisions were made prior to emasculation at the base of the lip. This blocked ca. 30 or 60% of the vessels entering the lip. The lip did not show any visible sign of turgor loss due to this treatment and control (non-emasculated) flowers were, at least during the first five days after the incision was made, not visibly affected. However, emasculation-induced coloration of the lip was significantly inhibited in both treatments (Fig. 1).

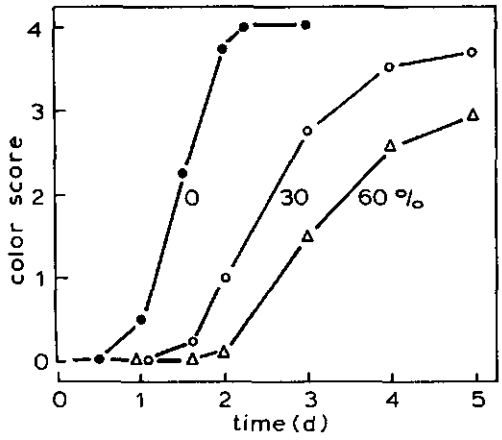

Fig. 1. Development of red coloration in *Cymbidium* lips following emasculation when 0% (●), ca. 30% (○), and ca. 60% (△) of the vessels entering the lip were blocked (n=10).

Inhibitor studies

After treatment of the column of non-emasculated flowers for 24 h with 4 µl/l ethylene the lip showed rapid coloration (Table 2). When NBD was applied simultaneously with ethylene, the ethylene-induced coloration of the lip was completely inhibited. Application of NBD alone was without effect in non-emasculated flowers (Table 2).

Lip coloration in emasculated flowers was completely blocked by treatment of the rostellum with AVG. Coloration was restored by an additional treatment of the AVG-treated flowers with ACC, ethylene, or ethephon. Coloration of the lip was not affected by treatment of the emasculated column with air or NBD (Table 2).

AVG was shown to effectively block the emasculation-induced accumulation of ACC in the column and this was not affected by an additional treatment with ethylene or ethephon. Similarly, ethylene did not induce accumulation of

ACC in columns of non-emasculated flowers (Table 2). In emasculated flowers and in flowers in which the column was treated with ethylene, ethephon, or a mixture of ethylene and NBD, a higher concentration of ethylene was found than in untreated intact flowers. Treatment of the emasculated flower with AVG prevented the rise in internal ethylene (Table 2).

Table 2. Effect of different chemicals on lip coloration, accumulation of ACC and internal ethylene concentrations in *Cymbidium* flowers.
AVG, ACC, and ethephon were applied onto the rostellum. Air, ethylene and NBD were applied to the upper half of the central column. AVG was given immediately after emasculation, thereupon ACC and ethephon were or were not applied. ACC and internal ethylene were analysed 20 h after the start of the 24 h treatment. n.d. = not determined.

Treatment	Emasculation	Time to coloration (d)	ACC content in central column (nmol/unit)	Internal ethylene concentration in the lip (µl/l)
Air	-	9	0.13	0.06
ethylene (4 µl/l)	-	1.5	0.14	0.16
NBD (4000 µl/l)	-	8	n.d.	n.d.
ethylene + NBD	-	9	n.d.	0.18
Air	+	1.5	1.31	0.25
NBD	+	1.5	n.d.	0.25
AVG (100 nmol)	+	12	0.09	0.07
AVG + ACC (5 nmol)	+	1.5	n.d.	n.d.
AVG + ethylene	+	2.0	0.14	0.20
AVG + ethephon (2 µl 1000 ppm)	+	1.5	0.13	0.17
LSD (5%)		3.6	0.08	0.04

Ethylene production of isolated flower parts

The pattern of emasculation-induced ethylene production in different flower parts is shown in figure 2. During the experimental period a significant change in ethylene production occurred in the central column but hardly any change was observed in the other flower parts (ovaries, lips, petals and sepals). From this experiment it could be calculated that approximately 90 to 95% of the total amount of emasculation-induced ethylene was derived from the central column. Considering that coloration of the lip was observed within 24 h after emasculation, these data indicate that coloration is not the result of an increased ethylene production in the lip.

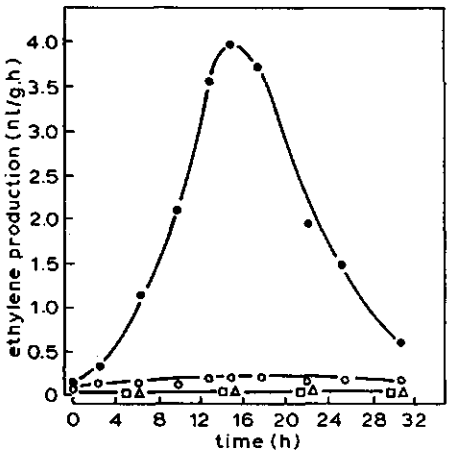

Fig. 2. Ethylene production of columns (●), ovaries (○), lips (□), and petals + sepals (△) isolated at different times after emasculation at t=0. Three excised flower parts were incubated per 30 ml glass vial and were flushed with ethylene-free air prior to closure. Lip coloration was apparent within 24 h after emasculation (n=2).

In situ **measurement of ethylene production**

The *in situ* measurements showed that, following emasculation, both the central column and the remaining portion of the flower produced significant amounts of ethylene. In fact, the remaining portion produced over 80% of the total amount of ethylene (Fig. 3A).

The same type of experiment was carried out with flowers that had been thoroughly treated with AVG (total uptake approximately 160 nmol) immediately after emasculation (which took place about 20 h before the start of the experiment). In this case, the ethylene production of the central column and the remaining portion was measured after addition of a small amount of ACC (2 nmol) onto the rostellum. Almost immediately after ACC application, the ethylene production of both the column and the remaining portion showed an increase comparable to the situation observed after emasculation (Fig. 3B). In this experiment, approximately 20% of the applied ACC was recovered as ethylene, the major part of which being produced by the remaining portion (68%).

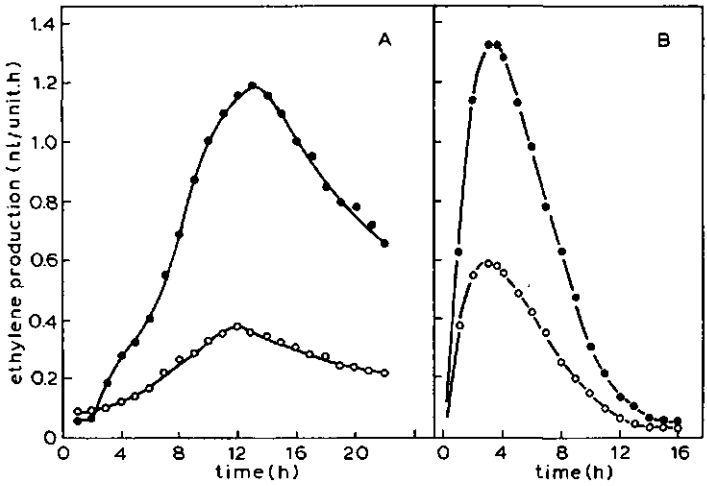

Fig. 3. Typical *in situ* measurement of ethylene production in the column (○) and the remaining portion (●) of a *Cymbidium* flower. The measurements were performed in a flow-through system.
A: Flower emasculated at t=0.
B: After application of 2 nmol ACC to the rostellar surface at t=0. Approximately 20 h before start of the measurements the flower was emasculated and treated with AVG.

Translocation of labeled ACC

The experimental set-up for *in situ* measurements was also used for the determination of the translocation of ^{14}C-ACC. About 55% of the total radioactivity recovered was present in the ethylene traps, the remaining portion producing 58% of the total amount of ethylene (Table 3). The extracts, including the holding solution, contained 45% of the activity recovered. Most of this activity (96.7%) was located in the upper part of the central column, *i.e.* at the site of application (Table 3).

A sample of the extract of the upper part of the column, run on cellulose TLC, showed two radioactive positions at R_f-values of 0.23 and 0.48, the latter coinciding with the ACC standard. According to Amrhein *et al.* (1982) the other component was N-malonyl-ACC. Integration of peak area revealed that about 87% of the activity in the top of the column was present as MACC.

Table 3. Radioactivity in ethylene traps and in different flower parts after application of radio-labeled ACC to the rostellum of an AVG-treated *Cymbidium* flower.
Data are from an individual flower; duplicate experiments yielded identical results.

Source of activity	Activity (Bq)	% of total activity recovered
Ethylene traps	6854	55
Extracts (incl. holding solution)	5564	45
	Activity (Bq)	**% of total activity in ethylene traps**
Ethylene from column	2901	42
Ethylene from remaining portion	3953	58
	Activity (Bq)	**% of total activity in extracts and holding solution**
Extract column top	5380	96.7
Extract column rest	60	1.1
Extract ovary (incl. peduncle)	12	0.2
Extract petals	10	0.2
Extract sepals	22	0.4
Extract lip	1	0.0
Holding solution	79	1.4

Early ethylene production in isolated lips

Lips, excised from emasculated and control flowers, were enclosed in small vials within approximately 30 s after excision. The accumulation of ethylene in the vials was determined by GC.

This experiment showed that the lips excised from emasculated flowers produced significant amounts of ethylene during the first 5 to 10 min after excision. Thereafter, the production ceased. Lips excised from non-emasculated flowers showed only a very low ethylene production during the experimental period (Fig. 4). From these experiments it could be calculated that the lips, at the time of excision (accumulation during the first min) produced ethylene at a rate of 1 to 2 nl/g.h.

Treatment of lips with ACC and ethylene

Isolated lips were placed with their cut base in low concentrations of ACC and ethylene production and coloration were determined at intervals.

The ethylene production was slightly stimulated by treatment with 5 μmol/l ACC. This only induced a faint pink coloration. At higher ACC concentrations, the ethylene production was more pronounced and this was accompanied by severe coloration (Fig. 5). From these experiments it may be argued that an increase in ethylene production up to 0.5 to 1 nl/g.h is required to induce a response comparable to the emasculation-induced coloration.

Both lips in non-emasculated and in AVG-treated emasculated flowers showed rapid coloration when treated with ethylene (Table 4). Similarly, isolated lips from non-emasculated and AVG-treated emasculated flowers showed a comparable development of coloration. This indicates that emasculation itself does not affect the response of lips to ethylene.

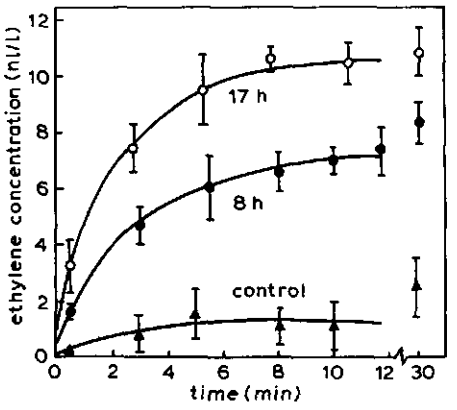

Fig. 4. Ethylene accumulation in 30 ml glass vials with six lips each. The lips were excised from control flowers (▲) and from flowers at 8 h (●) and 17 h (○) after emasculation. Data are means of 3 vials; vertical bars represent 2 x s.d.

Table 4. Effect of exogenous ethylene on development of lip coloration. Lips from intact (non-emasculated) and AVG-treated emasculated flowers were treated for 20 h with 4 μl/l ethylene while attached to the flower. Alternatively, the lips were excised from intact and AVG-treatred emasculated flowers at 15 h after start of the experiment, placed in water and treated for 20 h with different concentrations of ethylene. Color of the lips was evaluated at 24, 48 and 72 h after start of the ethylene treatment. AVG (100 nmol) was applied to the rostellum immediately after emasculation. Data are means ± s.d. of 10 flowers.

Treatment	Ethylene concentration (μl/l)	Color score		
		24 h	48 h	72 h
Lips attached to intact flowers	0	0	0	0
	4	1 ± 0	2.1 ± 0.2	3.2 ± 0.4
Lips attached to AVG-treated emasculated flowers	0	0	0	0
	4	1.1 ± 0.4	2.4 ± 0.5	3.4 ± 0.5
Isolated lips excised from intact flowers	0	0	0	0
	0.10	0	0	0
	0.22	0.1 ± 0.16	0.5 ± 0.42	0.6 ± 0.38
	0.41	0.1 ± 0.12	0.6 ± 0.60	1.6 ± 0.52
	0.82	0.4 ± 0.31	1.7 ± 1.20	2.3 ± 1.04
Isolated lips excised from AVG-treated emasculated flowers	0	0	0	0
	0.10	0	0.0 ± 0.12	0.0 ± 0.12
	0.22	0.1 ± 0.21	0.5 ± 0.40	0.6 ± 0.48
	0.41	0.1 ± 0.20	0.8 ± 0.61	1.6 ± 0.50
	0.82	0.4 ± 0.40	1.6 ± 1.46	2.5 ± 0.75

DISCUSSION

Emasculation of a *Cymbidium* flower leads to rapid coloration of the lip. This is the first sign of senescence in these flowers, later being followed by wilting of the entire perianth. When the lip is excised from the flower within 10 h after emasculation, coloration is completely prevented (Table 1). Blockage of part of the vessels entering the lip considerably delays coloration, indicating that some coloration-associated factor is moving in or out of the lip. These data are comparable to results in pollinated *Petunia* flowers where it was found that regions distal to small "windows" cut in the corolla tube show a delayed development of senescence symptoms (Lovell *et al.*, 1987).

Coloration of isolated *Cymbidium* lips was shown to be an effect of produced or applied ethylene (Woltering, 1989) and, therefore, it may be argued that the transported signal is related to ethylene. This may, for instance, involve the translocation of substances that stimulate the ethylene production in the lip (*e.g.* ACC, elicitors) or induce an ethylene effect (*e.g.* ethylene, sensitivity factor(s)). Determination of the ethylene production in different flower parts, isolated at different times after emasculation, revealed that only the production of the central column shows a significant increase. The other flower parts, including the lip, have only a very low production throughout the experimental period. It may therefore be concluded that the transported signal is not ACC but that rather ethylene itself may be translocated or the lip may become more sensitive to ethylene following emasculation. The latter would meet our expectations as the existence of (mobile) ethylene-sensitivity factor(s) has also been suggested in *Cyclamen*, *Petunia*,

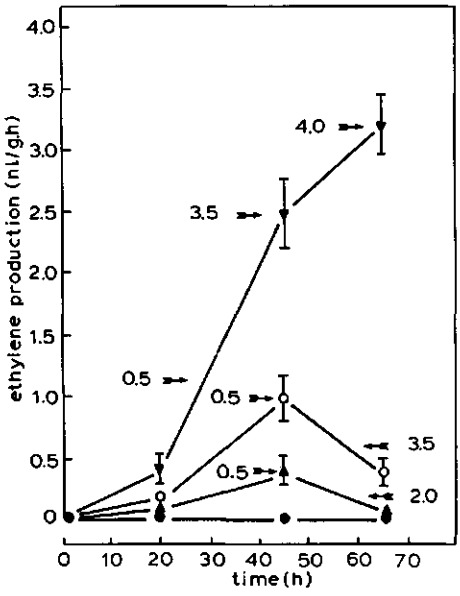

Fig. 5. Effect of ACC on ethylene production and coloration in isolated *Cymbidium* lips.
After excision, the lips were placed with their cut base in water (●), 0.005 mmol/l ACC (▲), 0.01 mmol/l ACC (0), or 0.02 mmol/l (▼) ACC.
Numbers indicate color score (see M + M).
Ethylene production was measured by enclosing the lips in 270 ml glass containers (12 lips each). Data are means (± s.d.) of 2 containers.

and carnation flowers (Halevy *et al.*, 1984; Hoekstra and Weges, 1986; Mor *et al.*, 1985; Whitehead and Halevy, 1989).

To further characterize the presumed transported senescence or wilting factor, the central column was treated with chemicals (Table 2). In emasculated flowers, coloration was effectively inhibited by AVG whereas treatment with air or NBD was without effect. Treatment of an AVG-treated flower with ACC restored the normal response. These data indicate that diffusion of ethylene out of the column and subsequently into the lip does not play a role and that column-produced ethylene may also not be involved in the synthesis of the wilting factor. Contrary to what may be concluded from the ethylene production measurements in isolated organs, the effects of AVG strongly suggest that in fact ACC may play a role in signal transduction.

Similar conflicting data were reported by Hoekstra and Weges (1986). These authors studied the ethylene production of different flower portions of *Petunia*, isolated at different times after pollination. During the early pollination-induced increase in ethylene production the wilting factor was shown to move into the corolla whereas no extra ethylene could be detected in this organ. In this case, the majority of the ethylene was produced by the gynoecium. They also found that AVG, applied onto the stigma, was a very effective inhibitor of pollination-induced senescence even when the entire style was removed before the AVG could have reached the corolla. This made Hoekstra and Weges (1986) conclude that, although direct proof was lacking, ACC may nevertheless be the transported wilting factor in *Petunia*. Pollination-induced corolla abscission in *Digitalis* was also not accompanied by an increased ethylene production in (isolated) corollas (Stead, 1985).

To analyse such conflicting results we used a sensitive laser photoacoustic detector for measurement of the *in situ* ethylene production of the central column and the remaining portion of the flower after emasculation and after addition of ACC to an AVG-treated flower. In addition, the transport of radio-labeled ACC was studied.

It was shown that, following emasculation, a simultaneous increase in ethylene production was observed in the central column and the remaining portion (Fig. 3A). The same was true after addition of ACC to the rostellum of an AVG-treated flower (Fig. 3B). Furthermore, addition of radio-labeled ACC to the column resulted in the production of radio-labeled ethylene by the column as well as the remaining portion (Table 3). In all these cases, more

than half of the total amount of ethylene was produced by the remaining portion.

Contrary to the results obtained with isolated organs, these data show that in the intact flower all the different organs may produce significant amounts of ethylene following emasculation. Furthermore, this ethylene may well be derived from ACC which was shown to be translocated from the site of application to the other parts of the flower (Fig. 3B).

To explain the lack of ethylene production in isolated flower parts it was argued that, if these organs have little ability to synthesize ACC but have the ability to convert ACC to ethylene, the endogenous amount of ACC may rapidly decrease upon excision. This in turn should result in a rapid decrease of the ethylene production. This was indeed proven to be true. During the first min after excision, the lips produced relatively high amounts of ethylene which ceased within 5 to 10 min (Fig. 4).

From these data it could be calculated that the lip, while attached to the emasculated flower, may produce ethylene at a rate of 1 to 2 nl/g.h, a rate that was found to be sufficient to induce coloration in isolated lips (Fig. 5). It is therefore concluded that translocation of ACC plays an important role in emasculation-induced coloration of the lip.

The possibility that ethylene itself may also be transported was also investigated. When the column of a non-emasculated flower is treated with ethylene, the lip shows rapid coloration. Similarly, application of ethylene or ethephon to an AVG-treated flower also leads to lip coloration (Table 2). This already indicates that ethylene may not act through the induction of ACC-synthase and this was verified by analysis of ACC in the columns.

Measurements of ethylene concentrations in the lips revealed that, similarly to concentrations in lips of emasculated flowers, an enhanced ethylene concentration was found in lips from flowers treated with ethylene or ethephon. The same was true for petals and sepals (data not shown).

The transport of ethephon is thought to be slow (Beaudry and Kays, 1988; Lavee, 1984) and it was verified that lips, excised from ethephon-treated flowers, in contrast to the columns, did not show an increased ethylene production (data not shown). This indicates that no ethephon was present in the lip and that ethylene, externally applied or internally derived from ethephon, may indeed be translocated within the flower.

The significance of this finding is evident as we recently found that the ethylene concentrations in columns during senescence of *Cymbidium* flowers may reach levels up to 15 µl/l (Woltering, 1990). Although presumably not a prerequisite during the early response to emasculation, the translocation of ethylene may play an additional role in inter-organ communication during senescence of these flowers.

Our results are not in line with data from Pech *et al.* (1987). These authors treated the *Petunia* style + stigma for 6 h with 2 µl/l ethylene and did not find any effect on wilting. An explanation may be that the translocation of ethylene is relatively slow and that a much longer application or a higher concentration is necessary to be effective.

To our knowledge, ethylene transport between different flower organs has not yet been reported, and the situation in *Cymbidium* flowers may therefore well be an exception. The relatively thick layer of epicuticular wax in these flowers may form a barrier for gas diffusion.

To investigate the possibility that besides ACC and ethylene an additional factor, that renders the lip more sensitive to ethylene, may be synthesized in response to emasculation, the lips of non-emasculated and AVG-treated emasculated flowers were treated with ethylene. Both in the case where the lips were treated while attached to the flower and in the case where the lips were treated after excision, the effect of ethylene on development of red coloration was remarkably similar (Table 4). This indicates that the sensitivity of the lip to ethylene was not changed by emasculation.

The effect of NBD on ethylene-induced coloration is difficult to explain. NBD, when applied simultaneously with ethylene, effectively inhibited coloration although the accumulation of ethylene in the lip was not affected. It may be concluded that, like ethylene, NBD is transported to the lip. However, if this were true, it should also have been effective in counteracting the effect of emasculation. But even at concentrations of up to 100,000 µl/l this was not the case (data not shown). An explanation may be that the translocation of ACC is much faster than that of ethylene and NBD. This, however, needs to be ascertained.

In conclusion, it was shown that emasculation leads to a localized production of ACC and subsequent translocation of ACC and ethylene within the flower. This causes a simultaneous increase in the evolution of ethylene by all the different flower organs. This early ethylene production induces coloration of the lip and may initiate the other senescence phenomena. These early events are very similar to those following pollination of flowers in general. As also no indications for the existence of an ethylene-sensitivity factor were found, it may be argued that also in pollinated flowers the translocation of stigma-produced ACC or ethylene is responsible for early wilting thereby questioning the validity of the "sensitivity factor" concept.

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CHAPTER 9

EFFECTS OF ACC, IAA, MONOCARBOXYLIC AND SHORT-CHAIN FATTY ACIDS, AND ELUATES FROM EXCISED GYNOSTEMIA ON COLORATION OF ISOLATED *CYMBIDIUM* LIPS

Ernst J. Woltering

SUMMARY

Eluates from excised gynostemia (central column) stimulated ethylene production and induced coloration in isolated lips. A similar effect was apparent after treatment of the lips with indole-3-acetic acid (IAA) or with 1-aminocyclopropane-1-carboxylic acid (ACC). Eluate-induced ethylene production and coloration were not inhibited by pretreatment of the lips with aminoethoxyvinylglycine. IAA-induced ethylene production and coloration were inhibited by pretreatment. This suggests that the increased ethylene production was derived from ACC taken up from the eluate. However, the amount of ACC in eluates was about one thousand times less than the lowest concentration needed to induce coloration in isolated lips.

Monocarboxylic and short-chain, saturated fatty acids generally did not have a significant effect on ethylene production, ethylene sensitivity or activity of the ethylene-forming enzyme. In addition, these acids did not greatly affect lip coloration, indicating that the effect of the eluate was not due to the presence of these substances. The results indicate that *in vitro* studies with excised floral parts do not yield relevant information concerning *in vivo* processes.

INTRODUCTION

During flower senescence, numerous substances may be translocated within the flower. Sugars and cytokinins, for instance, are known to be translocated from the petals to the ovary (Nichols, 1976; Veen and Kwakkenbos, 1984; Van Staden and Joughin, 1988). Similarly, nitrogenous compounds and phosphorus may also accumulate in the ovary (Harrison and Arditti, 1976; Hew *et al.*, 1989). Such translocations generally occur in a later stage of senescence and may be the result of a loss of sink strength of the senescing petals or of an increase in sink strength of the ovary.

In an early stage of senescence transport of substances between the floral parts may play a role in the initiation and coordination of the senescence processes. Especially the nature of the presumed pollination-associated, mobile wilting factor has been studied intensively. Gilissen (1976; 1977) noticed that, in *Petunia*, pollen tube growth or mechanical wounding of the stigma and style induced premature wilting of the flower and argued that the stigma may produce a signal that moves to the other flower parts. This mobile wilting stimulus was produced within 6 to 8 h after pollination or wounding of the stigma and eluates from pollinated or wounded styles were shown to possess wilt-inducing properties (Gilissen and Hoekstra, 1984).

Following pollination in *Petunia*, rapid increases in 1-aminocyclopropane-1-carboxylic acid (ACC)-synthase activity and ACC levels were apparent in the stigma (Hoekstra and Weges, 1986; Pech *et al.*, 1987). As pretreatment of the stigma with a minute amount of the inhibitor of ACC synthesis, aminoethoxyvinylglycine (AVG), completely blocked the wilt-inducing effect of pollination, it was argued that ACC may be the transported factor (Hoekstra and Weges, 1986). However, despite their wilt-inducing properties, eluates from pollinated or wounded styles did not contain appreciable quantities of ACC (Gilissen and Hoekstra, 1984).

Only very recently, the composition of the style eluate was analysed by gas chromatography-mass spectrometry and it was shown that a number of short-chain saturated fatty acids were present (Whitehead and Halevy, 1989a). These fatty acids seemed to have wilt-inducing properties and it was suggested that they were produced in the pollinated style and subsequently transported to the corolla. In the corolla, they are thought to render the tissue more sensitive to ethylene thereby causing an acceleration of the

wilting process (Whitehead and Halevy, 1989a; 1989b).

Also in many harvested fruits, the sensitivity to ethylene is believed to increase with time. This change in sensitivity is thought to trigger the change from system-I ethylene (low, basal level) to system-II ethylene (autocatalytic upsurge) (Yang, 1987). It involves the rapid induction of ACC-synthase activity by ethylene, which may result, for instance, from an increase in number or activity of ethylene binding sites or release from restriction by the gradual decline of an inhibitor.

Many commodities were shown to have a much longer life span when attached to the plant than when detached. Meigh *et al.* (1967) noticed that the ethylene production of detached apples was much higher than of those still attached to the tree and they speculated that the parent tree may supply an inhibitor of ethylene synthesis to the fruit. This so-called "tree factor" has also been suggested to play a role in ripening of other fruit species (Burg and Burg, 1964). Especially some varieties of Avocado do not ripen while attached to the tree. After detachment, rapid synthesis of ethylene takes place, and the fruit ripens. It was suggested that the "tree factor" inhibited the activity of ACC-synthase thereby keeping the fruit in a pre-climacteric stage (Sitrit *et al.*, 1986). The factor also occurs in such herbaceous plants as *e.g.* tomato (Sawamura *et al.*, 1978).

Another hypothesis to explain advanced senescence in detached fruits has been put forward by De Pooter *et al.* (1982, 1984). Catabolic processes in detached fruits due to the lack of nutrient supply, were thought to yield small carboxylic acids which were shown to be converted into ethylene. Ethylene produced through this alternative pathway (it was not inhibited by treatment of the fruits with AVG), was suggested to trigger the autocatalytic methionine-derived ethylene production and the accompanying ripening and senescence phenomena.

We recently studied the effects of emasculation, *i.e.* removal of the pollinia and the anther cap, on senescence of *Cymbidium* flowers. Emasculation was shown to induce red coloration of the lip, due to the action of ethylene. The initial events in emasculation-induced phenomena were found to be desiccation of the tissue just below the anther cap (the rostellum), subsequent production of ACC in the rostellar region, and the production of ethylene by the flower (Woltering *et al.*, 1988; Woltering, 1989; Woltering and Harren, 1989; Woltering, 1990a). *In situ* measurements of the emascu-

lation-induced ethylene production in different floral parts and studies with ^{14}C-labeled ACC revealed that ACC was rapidly translocated through the central column to the perianth where it was converted into ethylene. Ethylene itself was also shown to be translocated within the flower (Woltering, 1990b). From these studies it was concluded that in the intact flower, coloration of the lip is the result of ethylene and ACC translocation over a distance of several cm from the central column to the perianth.

Preliminary experiments revealed that isolated lips changed color when inserted in the eluate of an excised emasculated gynostemium (central column). It was hypothesized that, analogous to the *in vivo* situation, ACC may be translocated from the column to the eluate and subsequently be taken up by the isolated lip causing an increase in ethylene production and coloration. Alternatively, other mobile wilting factors may be involved in such an *in vitro* system.

Therefore, the effects of ACC, indole-3-acetic acid (IAA) and eluates from excised (emasculated) gynostemia on coloration and ethylene production of isolated lips were investigated. In addition, the effects of monocarboxylic and short-chain fatty acids on ethylene production, ethylene sensitivity, activity of the ethylene-forming enzyme (EFE), and on coloration in isolated lips was studied.

MATERIALS AND METHODS

Chemicals

Aminoethoxyvinylglycine (AVG) and 1-aminocyclopropane-1-carboxylic acid (ACC) were dissolved in water. Indole-3-acetic acid (IAA) was first dissolved in a small amount of ethanol and thereafter diluted with water. Propionic acid, butyric acid, octanoic acid and decanoic acid were first dissolved in a small amount of ethanol and thereafter diluted to the desired concentration with either a 7 mM phosphate buffer (pH 7) or a 7 mM citrate buffer (pH 4). The ethanol concentration in the final solution was 0.1% in all cases.
Buffer chemicals were purchased from Merck (Darmstadt, West Germany); the other chemicals were purchased from Sigma (St. Louis, USA).

Plant material and coloration study

Flower spikes of *Cymbidium* cv. Jacobi were obtained from a commercial grower and transported dry to the laboratory. The experiments were performed with excised central columns and lips from flowers cut from the middle region of the spike. Red coloration of the lips was visually evaluated each day and rated on a scale from 0 to 4 with a discrimination of about 0.5 using color prints (Woltering, 1989): 0 = original color; 1 = slightly pink coloration; 2 = pink coloration; 3 = red coloration; 4 = dark-red coloration.

All experiments were carried out under controlled environmental conditions of 12 h white light (15 $\mu mol/m^2.s$) and 12 h darkness; 60% relative humidity and 20°C throughout.

Description of the *in vitro* system

After excision, the cut base of the column was dipped in water, superficially dried with a paper tissue and the column was then placed in a small plastic vial with its base in 1 ml water. Some of the columns were emasculated and an isolated lip was immediately placed with its cut base together with the column in the 1 ml water. In most experiments, the lips were pretreated with either water or AVG by placing them with their cut base in an aqueous solution of the chemical for ca. 20 h prior to start of the experiment.

Measurement of ethylene production in excised columns and isolated lips

The excised column, still in the plastic vial, was placed in a 20 ml glass vial that was closed with a screw cap containing a septum. To ensure a low humidity inside the vial, $CaCl_2$ grains were also inserted. After ca. 1 h, the ethylene concentration in the headspace was analysed by gas chromatography (GC). The ethylene production of individual lips that had been treated with column eluate was determined by placing the lip, still in the plastic vial with eluate but without the excised column, in a 20 ml vial. After 3 to 4 h, the ethylene concentration was analysed by GC.

To test whether column-produced ethylene itself may affect the coloration of the isolated lip, the column and the lip, both individually placed in a small plastic vial with water, were enclosed for ca. 24 h in a 270 ml desiccator. To ensure a low humidity inside the desiccator and to prevent the accumulation of carbon dioxide, KOH grains were inserted.

The GC (Intersmat, Pavillion du Bois, France) was equipped with a flame ionization detector and a 2.5 m stainless steel column (inner diameter 4 mm) filled with aluminum oxide (Chrompack, Middelburg, The Netherlands). The minimum detection limit for ethylene was ca. 2 nl/l.

Coloration, ethylene production, ethylene sensitivity and EFE-activity in isolated lips following treatment with chemicals

Isolated lips were placed with their cut base in an aqueous solution of the chemicals and coloration was visually evaluated each day.

For measurements of ethylene production, the lips were transferred to a 270 ml glass jar (10 lips each). After incubation for 3 to 4 h, the ethylene concentration in the head space was analysed by GC.

For determination of the ethylene sensitivity, the lips were placed in the treatment solutions and after ca. 24 h they were transferred, in the treatment solutions, to a fumigation chamber for the treatments with ethylene. The ethylene treatments were carried out in the dark at 20°C. During the treatments, the carbon dioxide concentration was kept low and the oxygen concentration maintained at ambient level (Woltering and Sterling, 1986).

The *in vivo* EFE-activity in isolated lips was determined by measurement of the rate of conversion of applied ACC to ethylene. The lips were placed with their base in 2 ml of an aqueous ACC solution (10 mmol/l) in 20 ml glass vials. After two h the vials were flushed with ethylene-free air and closed. Approximately one h later, the ethylene concentration in the head space was measured by GC.

Determination of ACC and MACC in column eluates

The cut bases of excised columns were dipped in water, dried with a paper tissue and the excised columns were then placed with their cut base in 0.5 ml water. Thereafter, some of the columns were emasculated and approximately 20 h later the eluates were used for determination of ACC or MACC.

To study the translocation of applied ACC, whole flowers were excised, placed in water and emasculated. Immediately after emasculation 10 µl of an AVG solution (10 mmol/l) was pipeted onto the rostellum. After ca. 4 h, the columns were excised and placed in 0.5 ml water and different amounts of ACC were pipeted onto the rostellum. ACC and MACC were analysed in the eluates after ca. 20 h of incubation.

ACC was analysed in 0.3 ml of the eluate according to the method described by Lizada and Yang (1979), using internal standardization. Similarly, ACC was analysed after treatment of the eluate for 2 h with an equal volume of 6 N HCl at 100°C and subsequent neutralization with NaOH. The difference between the data obtained with the two procedures was taken as the amount of MACC.

RESULTS

Ethylene production of excised columns

Following excision, the ethylene production of the non-emasculated column was initially very low but showed a wound-induced increase after two to four h (Fig. 1). Thereafter, the production remained at this higher level for more than 24 h. The ethylene production of the excised emasculated column showed a much more pronounced increase that reached its maximum value at about 12 h after emasculation and showed a decline thereafter.

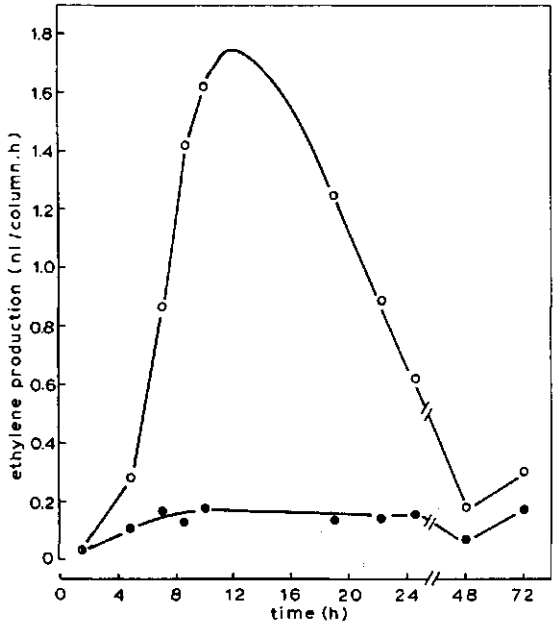

Fig. 1. Ethylene production of excised non-emasculated (●) and emasculated (o) columns. Data are means of 3 measurements.

Effects of eluates, IAA and ACC on coloration of isolated lips

Isolated lips did not show coloration when placed in water. However, when incubated together with an emasculated column, a faint pink coloration was generally visible within one day. This color developed into dark-red within the following two days. Lips incubated with non-emasculated columns also showed coloration, but this coloration was much less pronounced (Fig. 2, upper part). Pretreatment of the lips with AVG did not affect the development of red coloration when the lips were incubated together with an emasculated column. When lips were incubated with a non-emasculated column, a slower and less pronounced coloration was repeatedly observed after AVG treatment (Fig. 2, lower part).

Treatment of the lips with IAA (continuously) and ACC (for 24 h) caused rapid coloration. In the case of IAA, this coloration was completely absent when the lips were pretreated with AVG. AVG had no effect on coloration induced by ACC (Fig. 2).

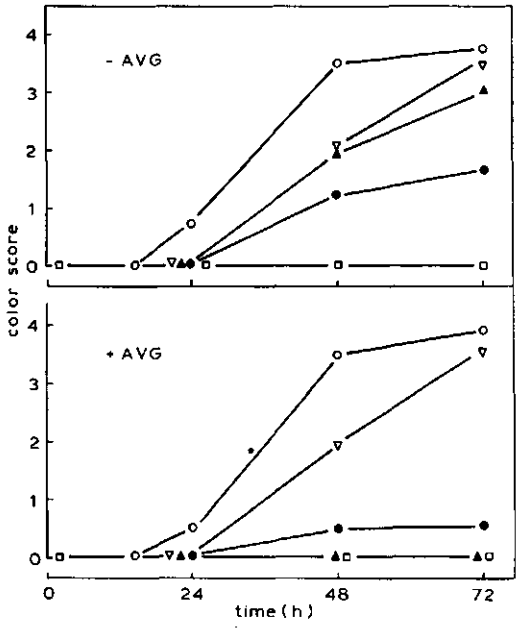

Fig. 2. Effect of column eluates, ACC and IAA on coloration of isolated lips. The lips were pretreated for ca. 20 h with either water (-AVG) or 0.5 mmol/l AVG (+AVG). Thereafter the lips were placed in water (□), in a solution of 0.2 mmol/l IAA (▲; continuously) or in a solution of 0.02 mmol/l ACC (▽; for 1 day and thereafter in water). Alternatively, the lips were incubated together with a freshly excised non-emasculated (●) or emasculated column (○) (n = 10).

Effect of eluates, IAA and ACC on ethylene production of isolated lips

Lips incubated in water showed a low ethylene production throughout the experimental period. Lips incubated with an emasculated column showed a small peak in their ethylene production and, although less pronounced, this was also observed in lips that were incubated with a non-emasculated column (Fig. 3). Treatment with IAA caused a pronounced increase in production, peaking at day two; treatment with ACC (for 24 h) caused a rapid increase in production peaking at day one.

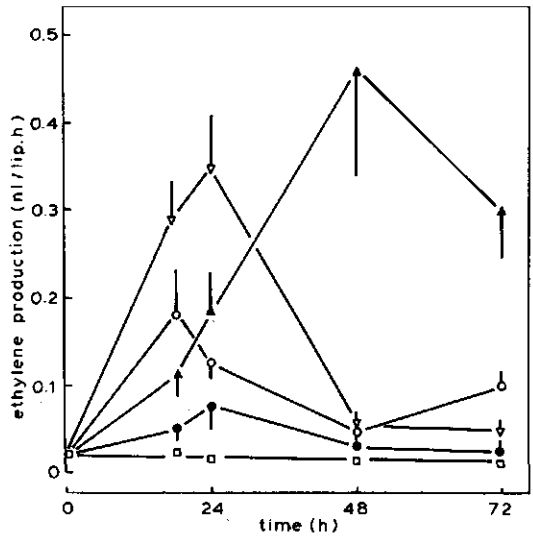

Fig. 3. Effect of column eluates, ACC and IAA on the ethylene production of isolated lips. The lips were placed in water (□), in a solution of 0.2 mmol/l IAA (▲; continuously) or in a solution of 0.02 mmol/l ACC (▽; for 1 day and thereafter in water). Alternatively, the lips were incubated together with an excised non-emasculated (●) or emasculated (o) column. Vertical bars represent 1 x s.e. (n = 5).

The effect of AVG pretreatment of the lips on the ethylene production induced by the eluates and by the different chemicals was determined after one day of incubation (Table 1). AVG was effective in counteracting the effect of IAA and that of the eluates from the non-emasculated column. However, AVG was much less effective in counteracting the effect of the eluate from the emasculated column, neither did it affect the ACC-induced ethylene production.

Table 1. Ethylene production of isolated lips placed for ca. 24 h in ACC (0.01 mmol/l), IAA (0.2 mmol/l), or incubated together with a non-emasculated or an emasculated column. The lips were pretreated for ca. 20 h with either AVG (0.5 mmol/l) or water prior to start of the experiment. Data are means of four measurements ± s.e.

Treatment	Ethylene production (nl/lip.h)		Effect of AVG (% inhibition)
	- AVG	+ AVG	
ACC	0.35 ± 0.052	0.35 ± 0.020	1.5
IAA	0.20 ± 0.034	0.02 ± 0.003	92
Non-emasculated column	0.08 ± 0.017	0.02 ± 0.008	76
Emasculated column	0.17 ± 0.027	0.17 ± 0.025	2

ACC and MACC in column eluates

The contents of ACC and MACC in eluates were determined after approximately 20 h of incubation. Recently, it was found that considerable breakdown of ACC could occur in vase water due to the action of micro-organisms (Overbeek and Woltering, 1990). Therefore, the amount of ACC and MACC was also determined in eluates containing 50 mg/l 8-hydroxyquinoline citrate (8-HQC), a known bacteriostatic compound (Marousky, 1969).

Only small amounts of ACC were detected in eluates from emasculated and from non-emasculated columns, irrespective of the presence of 8-HQC in the eluate (Table 2). In eluates from AVG-treated columns that were subsequently treated with different amounts of ACC (as a small droplet upon the rostellum) some extra ACC was only detected when the amount of applied ACC was more than 20 nmol (Table 2). In none of the cases was MACC detected in the eluates. Internal standardization with authentic ACC indicated full recovery of the applied ACC.

Effects of carboxylic and fatty acids on coloration, ethylene production, EFE-activity and ethylene sensitivity of isolated lips

Over a range of concentrations, the carboxylic and fatty acids, either applied at pH 4 or pH 7, did not greatly affect the average time to coloration of the lips (Table 3). At the highest concentration coloration was

considerably hastened in some cases, but this was accompanied by visible damage of the tissue at the cut base of the lip (water-soaked appearance), indicating phytotoxicity.

Table 2. Amount of ACC in eluates from excised columns after ca. 20 h of incubation, in three experiments (n = 6).
AVG (100 nmol) was applied to the rostellum at 4 h prior to excision of the column. ACC was applied to the rostellum at start of the incubation period.

Treatment of the column	ACC (pmol)		
	Exp. I (-HQC)	Exp. II (-HQC)	Exp. III (+HQC)
Non-emasculated	5.6	5.7	2.3
Emasculated (E)	5.4	7.0	9.7
E + AVG + water	4.1	1.6	n.d.
E + AVG + ACC (5 nmol)	9.3	n.d.[1]	n.d.
E + AVG + ACC (10 nmol)	3.6	0.5	n.d.
E + AVG + ACC (20 nmol)	11.3	n.d.	n.d.
E + AVG + ACC (100 nmol)	97.8	84.4	n.d.

[1] n.d. = not determined

Table 3. Effects of monocarboxylic and saturated fatty acids on coloration of isolated lips. The chemicals were applied either in a 7 mM citate buffer (pH 4) or a 7 mM phosphate buffer (pH 7).
The lips were placed for 5 days in these solutions and then transferred to water (n = 20).

Treatment	Concentration (mmol/l)	Time to coloration (d)	
		pH 4	pH 7
Buffer	-	> 25	> 25
Propionic acid	0.01	> 25	> 25
	0.1	> 25	> 25
	1.0	20	> 25
Butyric acid	0.01	> 25	> 25
	0.1	> 25	> 25
	1.0	> 25	> 25
Octanoic acid	0.01	> 25	> 25
	0.1	> 25	> 25
	1.0	5[1]	14[1]
Decanoic acid	0.01	> 25	> 25
	0.1	> 25	> 25
	1.0	4[1]	7[1]

[1] visible damage at base of the lip

Generally, the ethylene production of the lips was below the detection limit (< 10 pl/lip.h). Only at the highest concentrations of the applied chemicals was some stimulation of the production observed (Table 4). An increased ethylene production was accompanied by an earlier coloration of the lips. None of the chemicals had any effect on the EFE-activity on the second and the seventh day after the lips had been placed in solutions of 0.1 mmol/l of the different chemicals, irrespective of the pH at which they were applied (Table 5).

The effect of exogenous ethylene on coloration of the lips was evaluated after the lips had been in the different solutions for 24 h. The lips, still in the solutions, were treated for 24 h with different concentrations of ethylene. Thereafter, coloration was evaluated for two successive days. When compared to buffer controls, none of the chemicals, whether applied at pH 4 or pH 7, had any effect on the severity or the rate of ethylene-induced coloration (Fig. 4).

Table 4. Effects of monocarboxylic and saturated fatty acids on ethylene production of isolated lips after 2, 5, 9 and 13 days of treatment. The chemicals were applied either in a 7 mM citrate buffer (pH 4) or a 7 mM phosphate buffer (pH 7). The lips were placed for 5 days in these solutions and then transferred to water. Data are means of two jars with 10 lips each.

Treatment	Concentration mmol/l	Ethylene production (pl/lip.h)							
		pH 4				pH 7			
		d 2	d 5	d 9	d 13	d 2	d 5	d 9	d 13
Buffer	-	< 10	12	< 10	< 10	14	< 10	14	< 10
Propionic acid	0.01	< 10	< 10	< 10	< 10	< 10	< 10	11	< 10
	0.1	< 10	< 10	16	< 10	11	13	< 10	< 10
	1.0	38	36	53	24	19	16	13	10
Butyric acid	0.01	< 10	< 10	20	< 10	13	< 10	< 10	< 10
	0.1	11	< 10	14	10	< 10	< 10	11	< 10
	1.0	38	21	14	< 10	22	< 10	21	< 10
Octanoic acid	0.01	10	< 10	< 10	< 10	19	11	13	< 10
	0.1	< 10	< 10	< 10	< 10	22	< 10	13	< 10
	1.0	154[1]	-	-	-	< 10	< 10	59[1]	32
Decanoic acid	0.01	< 10	< 10	12	< 10	16	12	14	< 10
	0.1	< 10	< 10	< 10	< 10	26	< 10	10	< 10
	1.0	225[1]	-	-	-	42	37[1]	127	-

[1] Visible damage appeared at the base of the lip

Table 5. *In vivo* measurement of EFE-activity in lips placed in 0.1 mmol/l solution of different carboxylic or fatty acids on the second and the seventh day after start of the treatments. Data are means of four measurements.
Analysis of variance revealed no significant differences between the treatments.

Treatment	EFE-activity (nl ethylene/lip.h)			
	pH 4		pH 7	
	day 2	day 7	day 2	day 7
Water	1.42	0.25	1.42	0.25
Buffer	1.63	0.23	1.51	0.16
Propionic acid	1.51	0.24	0.99	0.38
Butyric acid	1.77	0.30	1.63	0.23
Octanoic acid	1.60	0.22	1.74	0.21
Decanoic acid	1.63	0.27	1.45	0.27

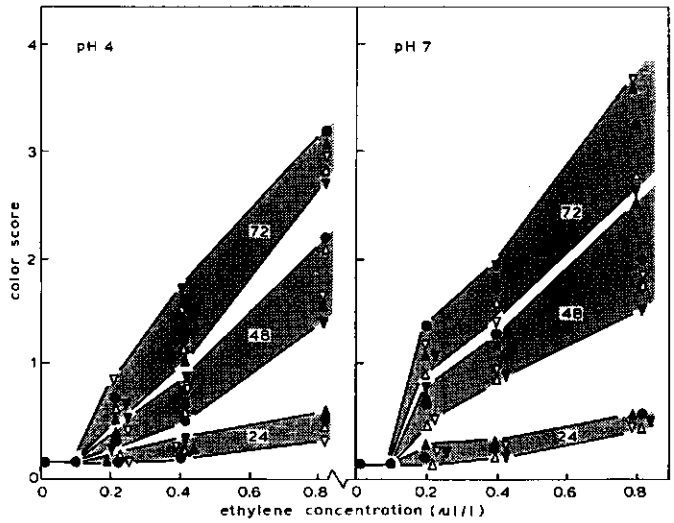

Fig. 4. Effect of ethylene (24 h) on coloration of isolated lips (measured 24, 48 and 72 h after start of the treatment) that were treated with buffer solution (●), propionic acid (△), butyric acid (▲), octanoic acid (▽) or decanoic acid (▼). The chemicals at a concentration of 0.1 mmol/l were applied in a citrate (pH 4) and a phosphate (pH 7) buffer.

DISCUSSION

Eluates from excised columns induced coloration in isolated lips and this effect was much more pronounced when the column was emasculated (Fig. 2). This indicates that a coloration-associated substance moved from the column to the aqueous holding solution and subsequently into the lip. Alternatively, the ethylene produced by the column may directly affect the coloration of the lip, as the lip and column were placed very close together. However, it is not likely that the relatively small amount of ethylene produced by the emasculated column (ca. 1 nl/h, Fig. 1) would give rise to an increased concentration in the vicinity of the lip. Furthermore, when a lip and an emasculated column, both individually placed in a small vial with water, were incubated together in a small volume, no coloration appeared despite the increased ethylene concentration in the atmosphere.

When the lips were placed in solutions of IAA or ACC, rapid coloration appeared. Both compounds were shown to stimulate the ethylene production of the lip (Fig. 3), IAA presumably by its effect on ACC-synthase activity (Yu *et al.*, 1979; Jones and Kende, 1979), ACC by its direct conversion into ethylene (Cameron *et al.*, 1979). Pretreatment of the lips with AVG completely prevented the IAA-induced coloration and ethylene synthesis, which shows that AVG was taken up by the tissue, that the applied concentration was very effective, and that coloration was a result of endogenously produced ethylene via the ACC-synthase pathway. In lips that were incubated with a non-emasculated column, AVG inhibited, though to a lesser extent, both coloration and ethylene synthesis. This indicates that at least part of the ethylene was derived from endogenously produced ACC.

In contrast, AVG had no appreciable effect on coloration and ethylene production in lips that were incubated with an emasculated column (Fig. 2). This implies that the major part of the ethylene production of these lips was not due to a stimulation of ACC-synthase activity. Rather, it could be a result of the conversion of absorbed ACC or unknown substances into ethylene.

Analysis of ACC in column eluates revealed that practically no ACC diffused and that also no appreciable differences in ACC content between eluates from emasculated and from non-emasculated columns were apparent (Table 2). On a concentration basis the eluates contained 5 to 10 nmol ACC/l, a concentration approximately 1000 times less than the lowest concentration

needed to induce coloration in isolated lips (Woltering, 1990b). The low concentrations of ACC in eluates are therefore not supposed to induce the increased ethylene production and coloration of the lips.

These observations are in disagreement with earlier findings. In the intact *Cymbidium* flower, ACC was shown to be translocated from the (emasculated) column to the perianth and it could be calculated that 0.3 to 0.4 nmol was exported from the column (Woltering, 1990b). This is approximately 100 times more than found in the eluates. Similar conflicting observations were made by other authors. Hoekstra and Weges (1986) argued that in the intact *Petunia* flower ACC was most probably the pollination or wounding-induced transported wilting factor. However, no ACC was detectable in eluates from pollinated or wounded styles despite their wilt-inducing properties (Gilissen and Hoekstra, 1984).

Sacalis *et al.* (1983) compared the effect of eluates from gynoecia, excised from fresh and from aged carnation flowers, on the senescence of isolated petals. These authors argued that excreted ACC may be the cause of the observed accelerated senescence as a significant difference between the levels of ACC in eluates from fresh and aged gynoecia was found. However, the senescence-stimulating effects of these eluates are most probably not due to the ACC as, on a concentration basis, only about 50 nmol ACC/l was present. When applied to isolated petals of the carnation cultivars Lena and White Sim such low concentrations of ACC did not have any effect on wilting (Woltering, unpublished).

It has been established that the addition of a small amount of ACC (2 to 5 nmol) to the rostellum of a *Cymbidium* flower previously treated with AVG induced coloration of the lip (Woltering and Harren, 1989). It was shown that the applied ACC was translocated within the flower (Woltering, 1990b). In fact, it could be calculated that over 10% of the applied ACC was exported from the column and converted into ethylene by the remaining portion of the flower.

Surprisingly, when applied to an excised AVG-treated column, ACC was found to be largely immobile as even after application of large amounts of ACC, only a minor fraction (~ 0.1%) was recovered from the eluate. This indicates that the *in vivo* translocation of ACC is not simply a diffusion process but that other transport mechanisms may be involved. Alternatively, the ACC may be oxidized by enzymes present in the eluate.

The above-mentioned observations indicate that the effect of the eluate on coloration of the lip was probably not due to a stimulation of the well-established ethylene biosynthesis route in higher plants (Yang and Hoffman, 1984). The question arises whether an alternative ethylene-biosynthesis pathway may be responsible for the increased ethylene production. Such an alternative route, based on the conversion of small carboxylic acids to ethylene, has previously been established in apple fruits (De Pooter *et al.*, 1982; 1984).

In flower senescence, a regulatory role for short-chain saturated fatty acids has recently been suggested (Whitehead and Halevy, 1989b). It was shown that these fatty acids render the tissue more sensitive to ethylene, thereby initiating the autocatalytic upsurge in ethylene production that induces their wilting. In eluates from pollinated *Petunia* styles, such substances were found to be present (Whitehead and Halevy, 1989a; 1989b).

Carboxylic and short-chain fatty acids have been suggested to play a role in the induction of seed dormancy and have been shown to possess other growth-inhibiting properties (Berrie, 1975; Lynch, 1980; Ulbright *et al.*, 1982a; 1982b). Recently, however, it was reported that the effect of these and related substances was greatly dependent on the pH at which they were applied. At their respective pKa values, the tested acids with chain lengths from C_2 to C_8 possessed strong dormancy-breaking activity in rice seeds whereas these acids had no effect at all when applied at pH values above their pKa (Cohn *et al.*, 1987).

The effects of fatty acids on the ethylene production of excised tissue from a variety of fruits and vegetables was reported by Hyodo and Tanaka (1982). These authors showed that octanoic and decanoic acids, in particular, were potent inhibitors of ACC-induced ethylene production and hypothesized that the acids would associate with membranes and thereby affect the activity of the ethylene-forming enzyme.

To test whether these substances may play a role in senescence of *Cymbidium*, isolated lips were treated with different concentrations of propionic, butyric, octanoic and decanoic acids at pH 4 and pH 7. At non-phytotoxic concentrations, none of the acids, irrespective of the pH at which they were applied, had any appreciable effect on ethylene production, ethylene sensitivity, or EFE-activity (Tables 4 and 5; Fig. 4). In addition, these substances did not greatly affect the incidence of coloration (Table 3) which

shows that the coloration-stimulating effect of column eluates was not due to the possible presence of these monocarboxylic or short-chain fatty acids. The results are therefore in disagreement with earlier published data (Whitehead and Halevy, 1989a; 1989b; Hyodo and Tanaka, 1982).

Lip coloration is the first visible sign of senescence in cut *Cymbidium* flowers (Woltering, 1990a). The present study clearly shows that especially eluates from emasculated columns possess "wilt-inducing" properties. The mechanism, however, may be different from that involved in lip coloration in the intact flower. This implies that *in vitro* studies with excised flower parts may have only a limited value for our understanding of the processes that occur *in vivo*.

The emasculation-induced formation of anthocyanins in the *Cymbidium* lip was found to be preceded by a vast increase in the activity of phenylalanine ammonia-lyase (PAL), the enzyme being *de novo* synthesized in response to ethylene (Woltering and Somhorst, 1990). The emasculation-induced synthesis of ACC in the central column, in turn, was suggested to be an effect of "endogenous elicitor(s)" released by the rostellum cells as a result of water stress (Woltering and Harren, 1989).

Upon infection with microbes, many plants show accumulation of antimicrobial compounds (phytoalexins), a process that is often accompanied by an increase in PAL-activity (Lawton, *et al.*, 1983). Although ethylene may in many cases participate in phytoalexin production, it is most probably not a prerequisite (Darvill and Albersheim, 1984). Endogenous elicitors, *i.e.* cell wall fragments have been implicated in the elicitation of phytoalexins in many tissues (Davis and Hahlbrock, 1987). As anthocyanins and phytoalexins share, at least in part, the same biochemical pathway, it is suggested that lip coloration in the described *in vitro* system is a result of an increased PAL-activity which is induced by endogenous elicitors that may escape from the rostellum region to the eluate. In the natural situation this hypothetical factor apparently plays no significant role as treatment of the emasculated flower with the gaseous inhibitor of ethylene action, 2,5-norbornadiene, completely prevents lip coloration while desiccation of the rostellum and subsequent production of the elicitors, is not affected by this treatment (Woltering *et al.*, 1988).

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CHAPTER 10

GENERAL DISCUSSION

In a great variety of harvested fruits and flowers, the capacity to respond to ethylene through autocatalytic ethylene production increases with age. This change in "sensitivity" may be regulated at various levels. For instance, at the level of ethylene binding or processing of the signal at the transcriptional or the translational level. Alternatively, sensitivity may be regulated at the level of the ethylene-biosynthetic pathway. An increase in responsiveness to ethylene may eventually lead to the induction of autocatalysis and to an acceleration of the senescence process already initiated. Whether this change in responsiveness occurs at the level of the biosynthetic pathway or at that of the receptor site is at present unknown. It may be due to a gradual release from restriction by the decline of an inhibitor or the production of an effector.

Of interest in this respect is the discovery that, in carnation petals, the cytoplasm may contain substances *i.e.* certain sugars and phenolic compounds that inhibit the activity of 1-aminocyclopropane-1-carboxylic acid (ACC)-synthase and the ethylene forming enzyme (EFE) (Mayak and Borochov, 1984; Itzhaki *et al.*, 1987). On the other hand, cell wall fragments have been shown to stimulate ethylene production by increasing the activity of ACC-synthase (Tong *et al.*, 1986). Various treatments, including pollination, water stress and ethylene are known to hasten senescence and the accompanying rise in ethylene production. Studying the effects of stress therefore provides a way to elucidate the nature of these factors.

In orchid flowers, emasculation by removal of the pollinia and anther cap, leads to an increased rate of senescence. Therefore, this treatment was taken as a model to study the biochemical changes preceding the rise in ethylene production that accompanies senescence. Potentially, this system is more suitable to work with than the pollinated flower, as pollen itself may contain representatives of all classes of hormones and other plant components (Barendse *et al.*, 1970; Van Staden, 1979; Arditti, 1979; Whitehead *et al.*,

1983; Yang *et al.*, 1985). Constituents released from the germinating pollen may interfere with the physiological processes under study, thereby complicating the interpretation of experimental results.

It was clearly shown that, in *Cymbidium* flowers, emasculation initially stimulates ACC-synthase activity in the top of the central column as a result of localized desiccation (chapters 6 and 7). It was argued that desiccation, or death of a limited number of cells, may give rise to the release of "elicitors" responsible for the increase in enzyme activity. The increase in ACC-synthase activity then leads to a transient increase in ethylene evolution not only from the central column but from all the different flower parts (chapter 8). Both ACC and ethylene were shown to be translocated within the flower and these factors were demonstrated to be responsible for the ethylene response observed in organs at considerable distance from the site of ACC synthesis (chapter 8).

From the literature, a number of cases are known in which the translocation of ACC within plants is suggested. In tomato plants, ACC was shown to be translocated through the xylem from the roots to the shoot following waterlogging (Bradford and Yang, 1980). Similarly, xylem transport of ACC was suggested to appear after infection of tomato plants with root-knot nematodes (Glazer *et al.*, 1984). Application of radio-labeled ACC to leaves of tomato plants leads to the accumulation of radioactivity in the roots, which indicates that ACC may also be transported through the phloem (Amrhein *et al.*, 1982). In *Cucurbita pepo*, localized application of ethephon was suggested to induce translocation of ACC, produced at the site of ethephon application, to distal tissues (Hume and Lovell, 1983).

ACC may also be transported between the different organs that compose a flower. In *Ipomoea nil* the anthers were suggested to be a source of ACC that may be translocated through the filaments to the corolla, thereby affecting flower-opening and senescence (Kiss and Koning, 1989). In addition, a role for ACC as a mobile wilting factor was also suggested in pollinated carnation and *Petunia* flowers (Reid *et al.*, 1984; Hoekstra and Weges, 1986).

The above-mentioned examples plus our own observations in *Cymbidium*, which actually prove that endogenously produced ACC is rapidly translocated within the flower (Chapter 8), suggest that ACC translocation may play an important role in the coordination of various developmental processes in plants or plant organs.

The assumption that the features of the plant hormone system may be comparable to those of the animal hormone system (*e.g.* localized synthesis, transport to the site of action and a concentration-dependent response) has been challenged over the years as most plant hormones are produced in many plant cells and small changes in concentration are thought to be relatively ineffective (Trewavas, 1981, 1982; Starling *et al.*, 1984). The concerted action of ethylene and its direct precursor, however, shows that localized synthesis and transport to the site of action is a necessary feature for the hormone to be effective in this case.

The question then arises which transport system allows ACC to escape from the actions of the ethylene-forming enzyme and the malonyl transferase. Considering the observation that ACC is very poorly translocated in excised columns (Chapter 9) it may be suggested that ACC is not simply translocated by diffusion through a concentration gradient in the apoplast but that other mechanisms are involved.

Although the transient increase in ethylene production in *Cymbidium* flowers following emasculation was readily detected by the laser photo-acoustic detector used (chapters 2 and 3), it can easily be overlooked when conventional methods for analysis of ethylene, *e.g.* a gas chromatograph equipped with a flame ionization detector, are used. In the case of the *Cymbidium* flower, emasculation leads to a minor change in ethylene production from about 0.02 nl/g.h up to 0.2 to 0.3 nl/g.h at its maximum.

This small and short-lasting change in ethylene production, however, was shown to have a pronounced effect on the internal ethylene concentration (chapter 7) and, as a consequence, on several biochemical processes. For instance, an increase in the activity of phenylalanine ammonia-lyase (PAL) and anthocyanin synthesis (chapters 4 and 5), EFE-activity (chapter 7), and membrane permeability (chapter 3) were apparent. This shows that very small changes in ethylene production may already have a significant effect on senescence, thereby meeting a third criterium of the animal hormone definition.

Without detailed kinetic data of the *in vivo* ethylene evolution from different parts of the flower, one might easily misinterpret the experimental data. This may lead to the suggestion that the ethylene response observed in flower organs other than the central column may be the result of an increased

ethylene sensitivity or may be due to the synthesis of unknown wilting factor(s) (chapter 8). It is therefore suggested that in those cases where a role for such factors is indicated, the observations may also be considered in terms of changing ethylene concentrations.

For instance, it was suggested that during senescence of carnation petals the basal part may transmit, besides ACC, an ethylene-sensitivity factor to the upper part (Mor *et al.*, 1985). This conclusion was based on the observations that isolated upper petal portions did not respond to treatment with either ACC or ethylene whereas the upper part in the intact petal showed severe wilting following treatment of the petal with ACC or ethylene.

However, we recently showed (Overbeek and Woltering, 1990) that a marked synergistic effect is observed when the upper petal part is treated with a combination of ACC and ethylene. It is therefore suggested that, in the intact petal, the basal part provides the upper part with both ACC and ethylene. Ethylene initially stimulates the activity of the ethylene-forming enzyme which is a prerequisite for an effective conversion of ACC into ethylene. We also showed that upper petal portions, that do not show a visible response to ethylene, are in fact very sensitive to ethylene as low concentrations already markedly increased the EFE-activity. It seems that experimental data may easily be interpreted in terms of changing sensitivities even though other, more plausible explanations will be sufficient. This is at least partly due to the lack of an appropriate ethylene-sensitivity assay.

In a recent paper, Whitehead and Halevy (1989a) described the nature of a presumed pollination-induced ethylene-sensitivity factor in *Petunia* flowers. These authors showed that following pollination, but also later, during natural ageing, there is an increase in the levels of, among others, the short-chain saturated fatty acids octanoic and decanoic acids in the corolla. It was shown that, following pollination, these fatty acids were produced in the style and transported to the corolla. Like pollination, treatment of the stigma with these substances was shown to increase the sensitivity of the flower to ethylene which explains their effect on senescence.

Treatment of *Cymbidium* lips with a range of concentrations of these compounds did not induce coloration, nor did they have an effect on the response to exogenous ethylene (chapter 9). It may therefore be concluded that these fatty acids have no physiological role in *Cymbidium* flowers. Alternatively, the experimental data may have been misinterpreted.

The sensitivity-assay used by Whitehead and Halevy (1989a; 1989b) ran as follows: flowers were pollinated, the stigma was treated with fatty acids or the flowers were left untreated while attached to the plant. At different times after start of the experiment, the flowers were excised and transferred to an environment with 2 μl/l ethylene and the change in corolla color from pink to blue was determined at intervals. In these experiments, the pollinated and fatty acid-treated flowers showed a faster change in color than the untreated ones. Hence, it was concluded that the corolla becomes more sensitive to ethylene as a result of pollination or treatment with fatty acids.

However, from their data (Whitehead and Halevy, 1989a; 1989b) it can be calculated that if the response to ethylene was expressed relative to the respective controls (*i.e.* the pollinated, fatty acid-treated, and control flowers that were not treated with ethylene), the sensitivity to ethylene was not at all affected as in all cases the longevity of the flowers was reduced by ca. 50% as a result of ethylene treatment. Taking into consideration that pollination or treatment with fatty acids markedly reduced flower longevity in their experiments, it is quite logical that an additional treatment with ethylene would further accelerate the senescence process.

This example again shows that the interpretation of experimental data in terms of changing sensitivities to ethylene is essentially wrong. Different ways of testing and expressing sensitivity may lead to different answers, while also no unambiguous definition of ethylene sensitivity exists.

In our own experiments with different *Petunia* varieties (unpublished) we were not able to reproduce the effects described by Whitehead and Halevy. Although pollination consistently induced premature wilting, octanoic and decanoic acids, up to ten times the amount used by the authors, had no effect at all on these flowers.

Iwanami *et al.* (1979) have shown that, among others, decanoic acid strongly inhibits the germination of *Camellia sinensis* pollen *in vitro*. It seems therefore unlikely that, following pollination, such acids would be produced in the stigma as they might prevent the fertilization process.

It is therefore at present not at all clear which mechanism is involved in pollination-induced premature senescence of *Petunia* flowers. Analogous to *Cymbidium*, it may be suggested that an early exposure of the corolla to endogenous ethylene derived from pollination-induced ethylene or ACC in the stigma initiates the wilting process in this organ.

From our studies with the emasculated *Cymbidium* flower a general concept can be derived concerning the effect of stress on the initiation of the senescence process and on the advanced occurence of the ethylene peak accompanying wilting (Fig. 1). Localized desiccation or cell death leads, possibly via the release of elicitors, to a transient increase in the production of ACC and ethylene in the top of the central column, *i.e.* at the site of desiccation. As both ACC and ethylene are translocated within the flower, all the flower parts are briefly exposed to endogenously produced ethylene. This immediately leads to the induction of several senescence-related enzymes (*e.g.* PAL, EFE) and has an effect on membrane permeability, which indicates that the senescence process has started. Also, in non-emasculated flowers, a minor increase in the basal level of ethylene is observed well before the more pronounced upsurge. This indicates that senescence is initiated by small changes in the ethylene production which may originate from locally produced ACC in a tissue apparently under stress.

Both in *Cymbidium* and in carnation flowers, but also in other harvested horticultural crops, a considerable lag period appears between a brief period of stress (including treatment with ethylene) and the increase in ethylene production that accompanies wilting. The question therefore arises in what way the early events may regulate the timing of the increase in ethylene production. Based on the results from *Cymbidium* flowers it may be hypothesized that the increased activity of the ethylene-forming enzyme following emasculation or treatment with ethylene plays a role by lowering the level of free ACC. In order to maintain homeostasis this may have a stimulating effect on the production of ACC. A similar effect may result from the increase in malonyl transferase activity which is often observed after treatment with ethylene (Yang, 1987). Alternatively, ethylene has been found to be one of the factors inducing *de novo* synthesis of ACC-synthase. However, this could not be demonstrated in *Cymbidium* flowers. An increased ACC-synthase activity will be accompanied by an increased ethylene production that may further accelerate the senescence or ripening process.

Support for this view can be found in the literature. Treatment of carnation and rose flowers with inhibitors of ethylene biosynthesis (amino-oxyacetic acid) and ethylene action (silverthiosulphate) inhibited the increase in membrane microviscosity and fresh weight loss at an early stage of senescence *i.e.* well before the autocatalytic upsurge (Veen and Kwakkenbos,

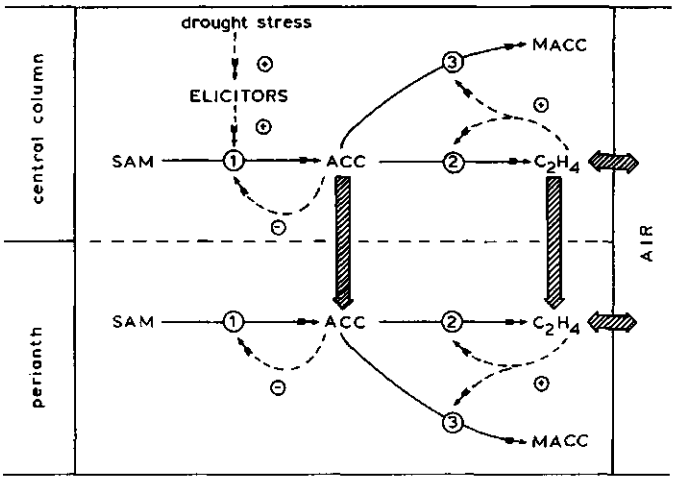

Fig. 1. Proposed scheme of the regulation of ethylene biosynthesis in different flower parts and the inter-organ communication.
Stress induces the release of elicitors in the central column which, in turn, stimulate ACC-synthase activity resulting in the production of ACC and ethylene. Both compounds are translocated to the perianth leading to the induction of EFE and the conversion of the translocated ACC into ethylene. The increased EFE-activity in both the column and the perianth may stimulate, via a negative feedback mechanism, the activity of ACC-synthase leading to a gradual increase in the production of ACC and ethylene.

——➔ Biochemical conversions
- - -➔ Stimulation (+) or inhibition (-) of indicated processes
▨ Translocation processes

(1) ACC-synthase
(2) Ethylene-forming enzyme
(3) N-malonyl transferase

1983; Mayak *et al.*, 1985; Faragher *et al.*, 1987). This indicates that the low basal levels of ethylene play a role in senescence. In tomatoes it appears that the increase in ACC and ethylene production preceding the ripening process starts in a discrete tissue *i.e.* the locular gel (Brecht, 1987). It was suggested that this early increase in ethylene production may initiate the autocatalytic upsurge observed at a later stage of ripening.

Similarly, in apple fruits, gradients in ACC levels were measured during ripening (Mansour *et al.*, 1986). The highest level was consistently detected in the core and the lowest level in the external cortex. As the increases during senescence appeared concomitantly in the different tissues these authors concluded that the climacteric was initiated independently in all the tissues. However, when we consider the possible transport of both ACC and

ethylene within the tissue, it seems quite reasonable to suggest that also in this case senescence (ripening) is initiated in a specific tissue, *i.e.* the core.

In *Petunia* flowers, it has been shown that a healthy, undamaged stigma is important in corolla longevity. One of its roles may be the prevention of the production of an abscission or wilting stimulus (Lovell *et al.*, 1987). In *Freesia* and in *Ipomoea nil* the anthers were recognized as a source of ACC and implicated in flower senescence (Spikman, 1987; Kiss and Koning, 1989).

The above-mentioned examples show that the initiation of senescence may, in many cases, start in a discrete tissue.

The initiation of senescence as a result of various types of stress is interesting from a practical point of view as many agricultural and horticultural products are severely stressed during storage and handling. Recognition of the sites initially affected by stress and further study on the involvement of the suggested elicitors is crucial for our understanding of plant senescence and may provide tools for use in breeding programs. Especially the presumed stress-induced release of small cell wall fragments (*e.g.* oligosaccharins) and their mode of action in stimulating the activity of ACC-synthase is an area in which little substantial information is available at present. The stress-induced increase in EFE-activity and its possible role in acceleration of the senescence process also merits further research and points to the possible measure of EFE as an early indicator of stress. Progress in studies on the properties of EFE, however, is slow as the enzyme has not yet been succesfully isolated.

The emasculated *Cymbidium* flower has proven to be a suitable model system for studies of the effects of stress, as the actual site initially affected by stress can be easily manipulated. In addition, we recently identified a number of clones that do not show the usual response to ethylene *i.e.* an increased rate of wilting and an earlier appearance of the upsurge in ethylene production. Following treatment with ethylene, these types of flowers show a delayed increase in ethylene production and lack the normal symptoms of senescence (inrolling of petals and shriveling). Instead, the flower slowly fades and becomes translucent. Further studies with these flowers will possibly yield valuable information on the mechanisms involved in the observed stress or ethylene-induced earlier occurrence of the autocatalytic upsurge in ethylene-sensitive commodities.

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SUMMARY

During storage and transportation, harvested crops are continuously exposed to all kinds of stress, such as desiccation and mechanical damage. These conditions are known to shorten shelf life and this is often associated with an earlier appearance of the sudden upsurge in ethylene production. It is doubtful whether this so-called autocatalytic ethylene production, although presumably necessary for the coordination and integration of the senescence process, is the trigger of senescence. Rather, changes in membrane properties or in ethylene sensitivity at an early stage of senescence may be responsible. An increase in the sensitivity to ethylene may, in turn, be an effect of the synthesis of so-called ethylene-sensitivity factors or the disappearance of inhibiting substances (chapter 1).

In *Cymbidium* flowers, as in other orchids, the stigma, style and stamens are united in an organ called the central column. Two pollinia, covered by an antherious tissue called the anther cap, are positioned on top of the central column. Removal of the pollinia and/or the anther cap (emasculation) is known to dramatically advance the senescence process. In nature, emasculation is carried out by insects, while searching for nectar in the flower, or by mice that feed on these apparently tasteful flower parts. As a result of suboptimal conditions during cultivation the anther caps may be abscised spontaneously, while during postharvest life the flowers may lose their anther cap due to handling.

Emasculation generally leads in about one day to red coloration of the labellum (lip), a modified petal differing in shape and color from the others. As the early effect of emasculation is very reproducible and easy to assess visibly, the *Cymbidium* flower was taken as a model system to study the biochemical changes that appear during stress(emasculation)-induced senescence. The purpose of the work described in this thesis, was to determine the factor(s) responsible for the advanced senescence and to analyse the interorgan relations during the senescence process.

From the literature it was derived that the negative effects of emasculation may be due to wounding. The disruption of a presumed cytokinin flow from the pollinia and the anther cap to the central column may also be involved. Concerning the communication between the floral parts, the biological precursor of ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC) as well as so-called ethylene-sensitivity factors (*e.g.* short-chain, saturated fatty acids) were suggested to be involved.

By using a very sensitive laser-driven photoacoustic detection system for ethylene we were able to show the existence of a small peak in ethylene production almost immediately after emasculation (chapters 2 and 3). This small and short-lasting increase in ethylene production appeared well before the visible symptoms (*e.g.* coloration of the lip), indicating a causal relationship.

Red coloration of the lip as a result of anthocyanin accumulation becomes visible within approximately one day after emasculation. This process is preceded by a pronounced increase in the activity of the key enzyme in phenylpropanoid metabolism, phenylalanine ammonia-lyase (PAL). A similar effect is apparent after treatment of the flower or the isolated lip with ethylene and inhibitors of ethylene synthesis and ethylene action greatly inhibit coloration. It is therefore concluded that lip coloration is regulated by ethylene (chapters 4 and 5).

Desiccation of the rostellum, an anther-derived tissue that is uncovered by emasculation, was found to be the primary factor in emasculation-induced ethylene production (chapter 6). Desiccation leads, possibly through the release of "endogenous elicitors", to an increase in the levels of ACC and malonyl-ACC in the top of the central column, which means that the endogenous elicitor locally stimulated the ACC-synthase activity (chapter 7). Determination of the ethylene production in different flower parts isolated at different times after emasculation, revealed that only the production of the central column shows a significant increase. The other flower parts, including the lip, have only very low productions (chapter 8).

Following emasculation, the lip does not show an increase in ethylene production but does show an ethylene effect (coloration). It may therefore be concluded that the sensitivity to ethylene has increased. Similar arguments were used by other authors to introduce a role for unknown sensitivity factors in senescence of *Petunia* and carnation flowers.

However, *in vivo* measurement of the ethylene production in the central column and the remaining portion of the flower showed that the major part of the ethylene (ca. 80%) is produced by the remaining portion and only a small amount by the central column *i.e.* at the site of ACC synthesis. In this way it was shown that ACC is rapidly translocated within the flower and that measurements in isolated flower parts do not yield valuable information concerning the *in vivo* ethylene production (chapter 8). The same is true for the translocation of ACC. Although ACC is rapidly translocated from the central column to the perianth, it is largely immobile in isolated columns

(chapter 9). With ACC as a transported intermediate, localized stress affects the senescence processes in all the flower parts. Ethylene itself was also found to be translocated within the flower and an additional role in the coordination of the senescence process is suggested (chapter 8). No indications for the existence of (mobile) ethylene-sensitivity factors were found and also no biological activity of the wilting and ethylene-sensitivity factors mentioned in the literature was apparent in this system (chapter 9).

The emasculation-induced ethylene production has a significant effect on the internal ethylene concentration in all the floral parts. As a result, an increase in the activity of different senescence-related enzymes, *e.g.* PAL and the ethylene forming enzyme (EFE), was observed (chapter 7). In addition, emasculation leads to an increase in membrane permeability (chapter 3). Although the ethylene production and the internal concentration both decrease to the initial level within one day, the EFE-activity is maintained at a high level for a longer period of time. This higher EFE-activity may stimulate, through negative feedback control rather than by its ethylene production, the ACC-synthase activity, leading to an earlier appearance of the more pronounced upsurge in ethylene production that accompanies senescence (chapter 10).

SAMENVATTING

Tijdens opslag en transport staan geoogste produkten voortdurend bloot aan verschillende vormen van stress, zoals vochtverlies en mechanische beschadiging. Dergelijke condities hebben doorgaans een sterk negatief effect op de houdbaarheid, hetgeen vaak gecorreleerd is met een eerder optreden van de sterke toename in de ethyleenproduktie. Mogelijk is deze zogenaamde autokatalytische ethyleenproduktie, alhoewel waarschijnlijk noodzakelijk voor de coördinatie en integratie van het verouderingsproces, niet de "trigger" van dit proces. Veranderingen van membraaneigenschappen of van de ethyleengevoeligheid in een vroeg stadium van de veroudering zijn mogelijk (mede) verantwoordelijk. Een verandering van de ethyleengevoeligheid zou weer een gevolg kunnen zijn van de synthese van zogenaamde ethyleengevoeligheidsfactoren of het verdwijnen van inhibitors (hoofdstuk 1).

In *Cymbidium*bloemen zijn, net als in andere orchideeën, de stempel, de stijl en de meeldraden verenigd in een orgaan dat centrale kolom genoemd wordt. Boven op de kolom liggen de twee stuifmeelklompjes welke weer afgedekt worden door de zogenaamde meeldraadkap. Verwijdering van de stuifmeelklompjes en/of de meeldraadkap wordt emasculatie genoemd en leidt tot een aanmerkelijke versnelling van het verouderingsproces.

Emasculatie kan in de natuur veroorzaakt worden door insekten die op zoek naar nectar de bloem inkruipen, of door muizen die zich voeden met deze blijkbaar smakelijke bloemdelen. Ook kunnen tijdens ongunstige teeltomstandigheden met name de kapjes spontaan afgestoten worden. In de naoogstfase kan een deel van de bloemen de kapjes verliezen als gevolg van de "handling".

Het effect van emasculatie is meestal al na één dag duidelijk zichtbaar doordat de zogenaamde lip, een opvallend en in kleur afwijkend bloemblad, sterk rood kleurt. Gezien het snelle, reproduceerbare en visueel goed waarneembare effect van emasculatie is de *Cymbidium*bloem gekozen als modelsysteem voor de bestudering van de biochemische veranderingen tijdens door stress (emasculatie) geïnduceerde veroudering. Het doel van het in dit proefschrift beschreven onderzoek was enerzijds het determineren van de factor(en), verantwoordelijk voor de versnelde veroudering, en anderzijds het vaststellen van de onderlinge relaties tussen de bloemdelen tijdens het verouderingsproces.

Uit de literatuur blijkt dat het negatieve effect van emasculatie mogelijk toegeschreven kan worden aan verwonding. Ook wordt het wegvallen van de cytokininestroom uit de stuifmeelklompjes en het kapje verantwoordelijk gesteld.

Voor de communicatie tussen bloemdelen worden zowel de precursor van ethyleen, 1-aminocyclopropaan-1-carbonzuur (ACC), als zogenaamde ethyleengevoeligheidsfactoren (bijv. verzadigde vetzuren) als mogelijke kandidaten aangemerkt.

Met behulp van een uiterst gevoelig laser-fotoakoestisch detectiesysteem voor ethyleen kon aangetoond worden dat er direct na emasculatie een geringe stijging van de ethyleenproduktie plaatsvindt (hoofdstuk 2 en 3). Dit ethyleenpiekje gedurende de eerste dag komt ruim voor het eerste zichtbare symptoom (lipverkleuring) en kan er dus verantwoordelijk voor zijn.

Roodverkleuring van de lip, hetgeen na ongeveer één dag waarneembaar is, wordt veroorzaakt door accumulatie van anthocyanen. Dit proces wordt voorafgegaan door een sterke stijging van de activiteit van het sleutelenzym in het fenylpropanoidmetabolisme, fenylalanineammonia-lyase (PAL). Omdat eenzelfde effect ook optreedt na begassing van de bloem of de geïsoleerde lippen met ethyleen en omdat remmers van de ethyleenwerking en -synthese het proces onderdrukken, kan geconcludeerd worden dat roodverkleuring gereguleerd wordt door ethyleen (hoofdstuk 4 en 5).

Uitdroging van het rostellum, een uit stempelweefsel bestaand orgaan dat bloot komt te liggen als het kapje wordt verwijderd, bleek de primaire factor bij de door emasculatie geïnduceerde ethyleenproduktie (hoofdstuk 6). Uitdroging leidt, mogelijk door het vrijkomen van "endogene elicitoren" tot een stijging van de ACC- en malonyl-ACC-gehalten in de bovenkant van de centrale kolom (hoofdstuk 7), hetgeen betekent dat de endogene elicitor plaatselijk de ACC-synthaseactiviteit stimuleert. Als de bloem op verschillende tijdstippen na emasculatie in onderdelen wordt verdeeld en hiervan de ethyleenafgifte afzonderlijk wordt bepaald blijkt dat alleen de centrale kolom een verhoogde ethyleenproduktie vertoont; de andere bloemdelen, inclusief de lip, produceren nauwelijks ethyleen (hoofdstuk 8).

Omdat de lip na emasculatie geen verhoogde ethyleenproduktie maar wel een ethyleeneffect vertoont, ligt de conclusie voor de hand, dat hier sprake is van een toegenomen gevoeligheid voor ethyleen. Dergelijke conclusies zijn ook door andere auteurs geformuleerd ter verklaring van de door stress of bestuiving geïnduceerde versnelde veroudering in anjer- en petuniabloemen.

In vivo ethyleenproduktiemetingen van de centrale kolom en de rest van de bloem toonden echter aan dat alle bloemdelen na emasculatie een verhoogde ethyleenproduktie vertonen. Het grootste gedeelte (ca. 80%) van het geproduceerde ethyleen bleek afkomstig van de bloemkroon en niet van de centrale

kolom waar de synthese van ACC plaatsvindt. Op deze wijze kon aangetoond worden dat ACC zeer snel binnen de bloem vervoerd wordt en dat ethyleenproduktiemetingen aan geïsoleerde onderdelen een volledig verkeerd beeld van de werkelijke situatie opleveren (hoofdstuk 8). Alhoewel ACC binnen de bloem snel van de centrale kolom naar de bloemkroon wordt vervoerd, kon geen translocatie aangetoond worden in experimenten met geïsoleerde kolommen (hoofdstuk 9). Dus ook in dit geval leverden metingen aan geïsoleerde onderdelen geen relevante informatie over de *in vivo* processen op. Locale stress kan dus via de translocatie van ACC invloed uitoefenen op het verouderingsproces in alle bloemdelen.

Naast ACC bleek ook ethyleen binnen de bloem vervoerd te worden en een additionele rol hiervan bij de coördinatie van het verwelkingsproces is waarschijnlijk (hoofdstuk 8). Er werden geen aanwijzingen gevonden dat (transporteerbare) ethyleengevoeligheidsfactoren in dit systeem een rol spelen. Tevens kon geen biologische activiteit worden aangetoond van de in de literatuur genoemde verwelkings- en ethyleengevoeligheidsfactoren (hoofdstuk 9).

De door emasculatie geïnduceerde stijging van de ethyleenproduktie heeft een aantoonbaar effect op de interne ethyleenconcentratie in alle bloemdelen. Als gevolg hiervan wordt de activiteit van verschillende met verwelking geassocieerde enzymen zoals PAL en het ethyleenvormend enzym (EFE) gestimuleerd (hoofdstuk 7). Tevens leidt emasculatie tot een toename van de membraanpermeabiliteit (hoofdstuk 3). Alhoewel de ethyleenproduktie en de interne concentratie al snel na emasculatie weer naar een laag niveau terugzakken, blijft de EFE-activiteit voor langere tijd op een hoog niveau. Mogelijk stimuleert deze verhoogde EFE-activiteit, via een negatieve terugkoppeling, de ACC-synthase activiteit, waardoor in geëmasculeerde bloemen de gepronconceerde stijging van de ethyleenproduktie tijdens de verwelking eerder op gang komt dan in niet geëmasculeerde bloemen (hoofdstuk 10).

CURRICULUM VITAE

De auteur van dit proefschrift werd te Vlissingen geboren op 22 november 1955. Na het doorlopen van de middelbare school volgde hij van 1975 tot 1979 een studie aan de Rijks Hogere Tuinbouwschool te Utrecht (technische differentiatie).

Van 1979 tot 1989 was hij werkzaam bij het Sprenger Instituut te Wageningen. Tijdens deze periode werd onder meer onderzoek verricht naar de effecten van ethyleen, in relatie met andere omgevingsfactoren, op de kwaliteit van diverse soorten snijbloemen, potplanten en *in vitro* cultures. Binnen dit kader werd tevens onderzoek verricht naar het effect van diverse chemicaliën op de ethyleensynthese en -gevoeligheid en op bewaar- en transporteerbaarheid van bovengenoemde produkten. In 1987 werd, in samenwerking met de Vakgroep Plantenfysiologie van de Landbouwuniversiteit, met het onderzoek gestart dat in dit proefschrift beschreven wordt.

Sinds september 1989 is hij aangesteld als onderzoeker plantehormonen bij het Agrotechnologisch Onderzoekinstituut (ATO) te Wageningen.