Physiological and Molecular effects of Thidiazuron and Ethylene on Leaf Yellowing and Rooting of Pelargonium *(Pelargonium zonale* hybrids) Cuttings

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Dedication

To my
Younger Brothers,
Wife,
and
Children
to be.

This thesis is made to inspire them to aspire to strive to achieve the highest level of education possible.

AMEN

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Abbreviations

ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	1-aminocyclopropane-1-carboxylic acid oxidase
ACS	1-aminocyclopropane-1-carboxylic acid synthase
AdoMet	S-adenosyl-methionine
ANOVA	Analysis of Variance
AVG	Amino-ethoxyvinylglycine
BA	6-benzylamino purine
BLAST	Basic local alignment search tool
bp	Base pairs
C_2H_4	Ethylene
cin	cytokinin-insensitive mutant
CPPU	N-(2-chloro-4pyridyl)-N-phenyl-urea
cross-talk	communication between two separate, linear signal transduction pathways that are simultaneously activated in the same cell
ctr	Constitutive triple response
DNA	Deoxyribose nucleic acid
DNAase	Deoxyribonuclease
dNTP	Deoxyribonucleotide tri-phosphate
DW	Dry weight
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetate
ein	ethylene insensitive
EMBL	European molecular biology laboratory
EREBPs	Ethylene Response Element Binding Proteins
ERF	Ethylene response factor
eto	ethylene overproducer mutant
ETR	Ethylene receptor gene
etr	Ethylene resistant mutant
FW	Fresh weight
GA	Gibberellin
IAA	Indole acetic acid
IBA	Indole-3-butyric acid

ipt gene	Isopentenyl transferase gene
LB medium	Luria-Bertani medium
NAA	Naphthalene acetic acid
NCBI	National center for biotechnology information.
Nr	Never ripe (LeETR3)
1-MCP	1-methylcyclopropene
RH	Relative humidity
RNA	Ribose nucleic acid
RNase	Ribonuclease
RT-PCR	Reverse transcriptase polymerase chain reaction
SAG	Senescence associated genes
SAM	S-adenosyl-methionine
STS	Silver thiosulphate
TAE buffer	Tris-Acetate-EDTA buffer
Taq	*Thermus aquaticus*
TDZ	Thidiazuron
Tris	tris-(hydroxymethyl) aminomethane
WC	Water content

Kurzfassung

Physiologische und molekulare Effekte von Thidiazuron und Ethylen auf Blattvergilbung und Bewurzelung bei Pelargonium-Stecklingen (*Pelargonium zonale* Hybriden)

Die Blattvergilbung ist ein wichtiges Problem bei Pelargionien-Stecklingen, da diese die Nacherntequalität negativ beeinflusst. Daher sind die Blattalterung, das Wachstumspotential und die Bewurzelungsfähigkeit wichtige Qualitätsmerkmale der Stecklinge. In dieser Arbeit sollte die Beeinflussung der Blattalterung durch Behandlungen mit TDZ, Ethylen und ABA oder eine Lagerung im Dunkeln untersucht werden, um mögliche praktische Anwendungen zu entwickeln, die zur Verbesserung der Stecklingsqualität bei *Pelargonium zonale* führen. Für die Untersuchungen standen fünf verschieden *Pelargonium zonale* Sorten ('Fire', 'Ganymed', 'Greco', 'Katinka' and 'Surfing') der Firma Selecta Klemm GmbH als Mutterpflanzen zu Verfügung. Die Stecklinge wurden nach dem Schneiden mit unterschiedlichen Konzentrationen von TDZ, Ethylen und ABA behandelt oder für 4 Tage bei Dunkelheit gelagert. Die Versuche wurden in einer vollständig randomisierten Anordnung mit zwei Wiederholungen und jeweils vier Stecklingen pro Behandlung durchgeführt. Die Messungen der Blattfarbe und die Bestimmung der Chlorophyllkonzetartion wurden bei allen behandelten Stecklingen durchgeführt. Die Entnahme der Blattproben für die molekulargenetischen Studien erfolgte, bevor die Stecklinge in einer Nährlösung (hydrophonisch) für einen Monat bewurzelt wurden. Für die molekulargenetischen Analysen mittels PCR und RT-PCR wurde DNA und RNA aus unterschiedlichen Organen der Sorten 'Katinka' und 'Ganymed' isoliert. TDZ Behandlungen führten bei allen Sorten zu einer signifikanten Verzögerung der Blattalterung. Die Lagerung der Stecklinge im Dunkeln und die Behandlung mit Ethylen oder ABA führten hingegen zu einer Beschleunigung der Blattalterung. Eine Hemmung der Blattalterung mittels TDZ konnte durch eine Erhöhung des Blatt-Hue-Wertes und eine Verringerung des Blatt-Chroma-Wertes nachgewiesen werden. Dagegen zeigten alle weiteren Behandlungen gegensätzliche Werte. TDZ führte außerdem, im Vergleich zu der unbehandelten Kontrolle, zu einer Erhöhung des Chlorophyllgehaltes. Eine Ethylenbehandlung (2 µl l^{-1}) führte bei allen Sorten zu einer Erhöhung der Bewurzelungsrate, jedoch zeigte sich auch eine Reduzierung weiterer Wurzelparameter. Die Lagerung im Dunkeln und die Behandlung mit ABA reduzierte die Anzahl der Wurzeln pro Steckling, die Wurzellänge, den Wassergehalt und das Trocken- und Frischgewicht. Die Zugabe von 4 µl l^{-1} IBA in die Bewurzelungsnährlösung führte bei der Sorte 'Ganymed' zu einer maximalen Bewurzelungsrate von 100%, erhöhte die Anzahl, die Länge, das Frisch- und Trockengewicht (%) und führte damit effektiv zu einer Revidierung der inhibierende Effekte von TDZ auf das Wurzelwachstum bei 3 Sorten. Durch den Einsatz von degenerierten Primern konnten drei DNA Fragmente aus genomischer DNA von 'Katinka' amplifiziert werden. Durch anschließende Sequenzanalysen gelang es, zwei neue, unvollständige, mögliche ACC Synthase Gene nachzuweisen, die als *PzACS3* und *PzACS4* bezeichnet wurden. Nach der Konstruktion von spezifischen Primern von bereits bekannten und neu isolierten ACC Synthase Genen und von Ethylenrezeptor Genen (ETR) wurden Expressionsanalysen mittels RT-PCR durchgeführt. So konnte eine starke Expression des Gens *PzACS3* und eine schwache Expression des Gens *PzACS4* in den Wurzeln nachgewiesen werden. Wohingegen das Gen *PzETR1* eine starke Expression in Wurzeln und Knospen zeigte. Aufgrund dieser gewebespezifischen Expression kann vermutet werden, dass die untersuchten Gene eine unterschiedliche Funktion bei der Regulation der Ethylenbiosynthese und Ethylenperzeption haben. Diese differentielle Expression konnte auch nach unterschiedlichen Behandlungen nachgewiesen werden. Außerdem zeigte sich eine Korrelation der Expression mit der Ethylenproduktion der Stecklinge. Eine exogene Ethylenbehandlung hatte jedoch keinen Einfluss auf das Expressionsmuster der analysierten Gene. ABA und die Lagerung im Dunkeln erhöhte die Expression der Gene *PzACS1* und *PzACS2*. Außerdem konnte erstmals die Induktion einer starken Expression des Gens *PzETR1* nach einer TDZ Behandlung nachgewiesen werden. Zusammenfassend (physiologisch und molekular) zeigen die Ergebnisse dieser Arbeit, dass die Blattvergilbung, die durch Ethylen, ABA oder Lagerung im Dunkeln bei *Pelargonium* Stecklingen induziert wird, durch eine TDZ Behandlung revidiert werden kann. Möglicherweise wird dieser TDZ Effekt durch die nachgewiesene Erhöhung der *PzETR1* Expression hervorgerufen, die dann zu einer reduzierten Ethylensensitivität führen und so die Blattalterung verzögern wird. Außerdem konnte gezeigt werden, dass die Wurzelinhibierung durch die TDZ Behandlung durch IBA in die Bewurzelungsnährlösung revidiert werden kann.

Schlagwörter: ACC-Synthase, Ethylenrezeptor, Genexpression, Blattalterung, *Pelargonium zonale*, Phytohormone, Lagerung, TDZ

Abstract

Physiological and Molecular effects of Thidiazuron and Ethylene on Leaf Yellowing and Rooting of Pelargonium *(Pelargonium zonale* hybrids) Cuttings

Leaf yellowing is a major problem in *Pelargonium* cuttings and leads to high post harvest losses. Hence, the absence of leaf senescence symptoms and the capacity of cuttings to continue growth of initiated roots are vital quality attributes. The effects of treatments with TDZ, ethylene and ABA or dark storage during leaf senescence process were studied to possibly come up with a potential commercial approach for improving post harvest quality of *Pelargonium* cuttings. Five new *Pelargonium zonale* cultivars ('Fire', 'Ganymed', 'Greco', 'Katinka' and 'Surfing') from Selecta Klemm GmbH were grown into stock-plants. Cuttings were treated after harvest with various levels of TDZ, ethylene, ABA or stored in the dark for 4 days. Then arranged in a completely randomized design comprising of 2 replications per treatment of four cuttings per replication and evaluated for leaf colour and chlorophyll content. Leaf samples were taken for molecular studies to examine gene expression after treatments before cuttings were hydrophonically for one month rooted in greenhouses. DNA and total RNA were isolated from 'Katinka' and/ or 'Ganymed' tissues for PCR and One step RT-PCR, respectively. TDZ treatment markedly delayed the onset of leaf senescence in all cultivars. Storing cuttings in dark and treating them with ethylene or ABA hastened the onset of leaf yellowing while TDZ retarded leaf yellowing. TDZ treatment increased leaf hue and decreased leaf chroma whereas the reverse was true for other treatments because the leaves turned yellow. TDZ treated leaves had high chlorophyll contents, while levels declined in the untreated controls. Ethylene (2 $\mu l\ l^{-1}$) increased rooting percentage in all cultivars but reduced other root parameters. Dark storage and ABA reduced number of roots per cutting, reduced root length, root water content, fresh and dry weights. Applying 4 $\mu l\ l^{-1}$ IBA in nutrient solutions induced maximal (100%) root induction in 'Ganymed', increased numbers and length of roots, fresh and root dry matter (%) accumulation and effectively offset the inhibitory effect of TDZ on root formation for cultivars 'Fire', 'Katinka' and 'Ganymed'.

Degenerate primer pair was used to amplify three DNA fragments using genomic DNA from 'Katinka' leaves. Sequence analysis of two novel partial putative ACC synthases led to their characterisation and designation as *PzACS3* and *PzACS4*. *PzACS3* is 590 bp long with 374 bp coding region and two introns while *PzACS4* is 745 bp long with 374 bp exon and two introns too. Gene-specific primers for the new ACC synthase, other ACC synthase and ethylene receptor (ETR) genes were synthesized. Expression studies were done using a One step RT-PCR. *PzACS3* and *PzACS4* transcripts were expressed and undetectable in roots, respectively, while *PzETR1* was strongly expressed in roots and flower buds. Tissue specific gene expression patterns suggest they have different roles in ethylene biosynthesis and signaling. Also, transcripts of these genes were induced in a treatment-specific fashion and correlated positively with ethylene production by cuttings after 4 days in various treatments except for 2 $\mu l\ l^{-1}$ ethylene which had inhibitory effect. Ethylene slightly down regulated accumulation of *PzACS1* mRNAs. ABA and dark storage increased expression of *PzACS1* and *PzACS2* mRNAs, respectively. Moreover, for the first time, TDZ was shown to strongly induce expression of *PzETR1*.

Taken together (physiological and molecular), these results suggest leaf yellowing in *Pelargonium* cuttings was due to either stress-induced ethylene that occurs after dark storage or ABA treatment and TDZ was able to antagonise their deleterious effects, by increasing the amount of ethylene receptors via up-regulation of *PzETR1* transcripts, thus reducing sensitivity of leaves to ethylene with the concomitant beneficial effect of delaying the onset of senescence. Also, inhibition of rooting by TDZ was effectively offset by applying IBA in rooting solutions through induction of adventitious roots.

Key words: ACC synthase, Ethylene receptor, Gene expression, Leaf senescence, *Pelargonium zonale*, Phytohormones, Storage, TDZ

1.0 General Introduction

Overview

Pelargonium zonale hybrid (syn. *Pelargonium x hortorum* L. H. Bailey), also known as storksbill or geranium, is one of the most popular potted and bedding plants in Europe and North America (Serek *et al*., 1998). *Pelargonium zonale* hybrid had a global annual sale of $ 700 million (Canadian dollars) in 2000 (Mithila *et al.,* 2001). Also, it is one of the popular floricultural crops in Germany. In 2000, Germany recorded a total production of 117 million mature plants comprising of 113 balcony plants and 4 million potted flowering plants (Statistisches Bundesamt, 2001). *Pelargonium zonale* hybrids are grown for their colourful and showy flowers, scented foliage and exotic leaf shape. Furthermore, NBV/UGA flower auction in Straelen, Germany traded a total of 7.2 million *Pelargonium zonale* plants valued at an average price of € 0.82 per plant (NBV/UGA Geschäftsbericht, 2001). This was in contrast to the USA where the market of *Pelargonium* cuttings was valued at $ 111 million in 2003 (USDA, 2003: www.ers.usda.gov).

The genus *Pelargonium* belongs to the family *Geraniaceae* and nearly all of its 280 species originated from South Africa where they are naturally found growing in dry, hot habitats with sandy or rocky soils and/ or sand dunes (Van der Walt, 1977). The commercially important *Pelargonium* species have been categorised into four major groups: *zonale* geraniums (*Pelargonium* x *hortorum* L. H. Bailey), regal *Pelargonium* (*Pelargonium x domesticum*), ivy geraniums (*Pelargonium peltatum*) and scented geraniums (*Pelargonium sp.*) (Mithila *et al*., 2001). These species have been cultivated since 17th century (Huxley *et al*., 1992). The species *zonale* was first introduced to Europe in 1609 (Ewart, 1981).

Pelargonium is an annual or perennial herb with entire, lobed or dissected leaves, usually with prominent stipules at the base of the petiole. Flowers are borne in umbels on a peduncle, which may be terminal on the stem, axillary to the leaves or opposite (Huxley *et al*., 1992). The *zonale* geranium is probably one of the most beautiful of the flowering *Pelargonium* and is mainly propagated through cuttings. The number of plants produced from cuttings was almost equal to the number produced from seed, but the value of plants grown from cuttings was almost double that from seed (Berninger, 1993). Also, propagation by seed is restricted

because *Pelargonium* hybrids are highly heterozygous plants and seed propagation usually leads to the segregation in subsequent progenies (Mithila *et al*., 2001).

Absence of senescence symptoms in the leaves of *Pelargonium* cuttings, their capacity to initiate roots and continued growth of initiated roots is an important quality attribute (Purer and Mayak, 1988). This is because when cuttings undergo senescence, they show reduced rooting percent and number of main roots. Cuttings of high quality are essential in the highly competitive market. Moreover, senescing leaves turn yellow and are more prone to infection by diseases such as *Botrytis*. This in turn affects the rate of development and survival of new plants that take longer to get established and start vigorous growth (Purer and Mayak, 1988). Also, application of fungicides and more handling measures tend to increase cost of production and are therefore, undesirable in the competitive world market (Purer and Mayak, 1988).

1.2 Statement of the problem

European growers have been looking for a reliable and economical method for the storage of *Pelargonium* cuttings, in order to meet early spring demand (Paton and Schwabe, 1987). *Pelargonium* cuttings and other tropical foliage plants are imported from Africa, Latin America, the Canary Islands or the Middle East. They are subsequently rooted and finished in Europe (Serek *et al*., 1998). The above named areas are climatically favourable for cutting production and have the added advantage of a ready supply of labour and land, which are cheap as compared to Europe (Serek *et al*., 1998). The delivery process can take between 4 to 14 days. However, these cuttings are exposed to adverse conditions during shipment, such as exposure to ethylene, low humidity and long hours of darkness (Purer and Mayak, 1989) that induce senescence (Behrens, 1988). Leaf senescence is apparent as leaf yellowing due to chlorophyll loss. It is a common problem in *Pelargonium*, chrysanthemum (Purer and Mayak, 1989), *Alstroemeria* (Mutui *et al*., 2001), tulip, roses (Halevy and Mayak, 1981), and lillies (Han, 1997) and leads to high post harvest losses. Consequently, the maintenance of green colour and the altered potential of cuttings to initiate roots are important quality attributes in plant cuttings.

Leaf yellowing is a complex physiological process that may result from one or several inducers. Also, it is thought to be under control of phytohormones (Weaver *et al*., 1998).

Ethylene, ABA and darkness have been implicated as important factors in promoting leaf senescence (Nooden, 1988; Purer and Mayak, 1989; Becker and Apel, 1993). Additionally, these factors inflict economic loss. Senescing leaves are readily recognisable by a characteristic yellowing, starting at the veins and extending outwards, resulting in a loss of chlorophyll (Quirino *et al*, 2000). This in turn leads to a rapid decline in photosynthesis, as chlorophyll is lost. Consequently, rooting of the cuttings is delayed (Purer and Mayak, 1989). Smart (1994) proposed that the decrease in photosynthesis below a certain threshold level might function as a signal to induce senescence. In addition, chlorotic leaves not only reduce acceptability of cuttings but also promote susceptibility to *Botrytis*, which readily spreads within shipping containers (Carrow and Bahnemann, 1980).

Leaf senescence and chlorophyll degradation can be reduced or eliminated in a wide range of species by application of cytokinins (Thimann, 1980). Cytokinins are powerful inhibitors of leaf senescence (Gan and Amasino, 1996). Pulsing *Alstroemeria* with benzyladenine (BA) effectively reduced leaf yellowing and improved its post harvest vase life (Mutui *et al*., 2001, 2003; Whitman *et al.,* 2001) and other plant species (Halevy and Mayak, 1981). Recently, Thidiazuron (TDZ), a substituted phenyl urea with powerful cytokinin-like activity, has been reported to be very effective in preventing leaf yellowing and retarding chlorophyll degradation in *Alstroemeria* cut flowers (Ferrante *et al.*, 2002a), poinsettia (King *et al*., 2001), cut tulips and cut chrysanthemum (Ferrante *et al*., 2003). But, TDZ treatment inhibited rooting and promoted lateral shoot elongation in cut chrysanthemum ‘Regan giallo’ (Ferrante *et al.,* 2003), thus limiting its’ practical use. Also, 1-methylcyclopropene (1-MCP) retarded storage-induced leaf yellowing in *Pelargonium* cuttings (Serek *et al*., 1998). Therefore, in the current study, TDZ was used to prevent leaf senescence in *Pelargonium* cuttings. Thus exogenous auxins (IBA) were added into nutrient solutions to alleviate a TDZ rooting inhibition effect, since auxins are known to universally induce adventitious roots (Kelen and Ozkan, 2003).

1.3 Objectives

Overall goal

To elucidate physiological and molecular mechanisms of TDZ (cytokinin) that leads to a delay in the onset of leaf senescence. This was geared towards coming up with a potential commercial approach for improving the post harvest quality of *Pelargonium* cuttings.

Specific goals

1. To determine the effects of senescence inducers (ethylene, ABA and dark-storage) on leaf yellowing and rooting ability of *Pelargonium* cuttings.
2. To counteract the adverse effects of senescence inducers with senescence retardant (TDZ) via extension and improvement of post harvest quality of the cuttings.
3. To determine if auxins (IBA) could alleviate TDZ rooting inhibition effect to enhance the practical use of TDZ.
4. To investigate the expression of ethylene biosynthesis [*ACC* synthase and ethylene receptor (*ETR1*)] genes in various *Pelargonium* organs and after dark-storage or exogenous application of ethylene, ABA and TDZ treatments.

2.0 Effects of Thidiazuron, Ethylene, Abscisic acid and dark Storage on leaf yellowing and rooting of *Pelargonium zonale* hybrid cuttings

Abstract

The effects of post harvest treatments with TDZ, ethylene, ABA or dark storage of *Pelargonium* cuttings were investigated. TDZ treatment markedly delayed the onset of leaf senescence in cultivars 'Fire', 'Ganymed', 'Greco', 'Katinka' and 'Surfing'. Storing cuttings in the dark for 4 days and/ or treating them with 2 µl l^{-1} ethylene or 100 µM ABA hastened the onset of leaf yellowing, while 5 µM TDZ retarded leaf yellowing. TDZ increased leaf hue values and decreased leaf chroma values because TDZ-treated leaves remained green. In contrast, applying ethylene, ABA or dark storage decreased leaf hue and increased leaf chroma because the leaves turned yellow. TDZ treated leaves had high chlorophyll content, while levels declined in the untreated controls. Ethylene at 2 µl l^{-1} significantly increased rooting percentage (%) in all cultivars, but reduced other root parameters investigated. Short-term dark storage and 100 µM ABA reduced the ability of cuttings to continue growth of regenerated roots. This effect was evidenced by reduced number of roots per cutting, root length, water content, fresh and dry weights. Applying 4 µl l^{-1} IBA in the rooting solutions induced maximal rooting proportion in 'Ganymed', increased numbers and length of roots, fresh weight and root dry matter (%) accumulation in all cultivars. IBA effectively offset the inhibitory effect of TDZ on root formation. These results suggest leaf yellowing in *Pelargonium* cuttings was due to either stress-induced ethylene that occurs after dark storage or ABA treatment. However, TDZ was able to antagonise their deleterious effects with the concomitant beneficial effect of delaying the onset of senescence.

Key words: Leaf senescence, *Pelargonium zonale*, Phytohormones, Postharvest, Rooting, Storage

2.1 Introduction

2.1.1 Overview

Natural leaf senescence, also known as 'senescence syndrome', is a complex, genetically determined cell death program. It is characterised by a decline of macromolecules such as total protein, RNA levels, membrane lipids and chloroplast degradation (Weaver *et al.,* 1998). This catabolic process enables the plant to salvage and redistribute carbon and nitrogen into the growing tissues (Bleecker, 1998). The most visible symptom of senescence is a loss of chlorophyll pigments and concomitant yellowing of the leaves. The initiation and progression of leaf senescence can be influenced by various external factors such as extremes of temperature, moisture, pathogens, light intensity and duration (Chandlee, 2001; Smart, 1994; Weaver *et al.,* 1998). Internal senescence-inducing factors appear to be hormonal in nature (Chandlee, 2001). Conversely, phytohormones and certain forms of stresses hasten or repress senescence.

2.1.2 Ethylene

Ethylene is a gaseous plant hormone synthesised in all cells and moves freely through plant tissues (Yang and Hoffman, 1984). *Pelargonium* is an ethylene-sensitive flowering plant (Woltering, 1987). The response of *Pelargonium* to ethylene is dependent on concentration, duration of exposure, temperature and stage of development (Dole and Wilkins, 1999). Some of senescence-symptoms caused by ethylene in *Pelargonium* are leaf yellowing, leaf drop, bud abortion and bud abscission (Serek *et al*., 1998). However, 1-methylcyclopropene (1-MCP), a gaseous compound, completely inhibits ethylene effect and appears to bind irreversibly to the putative ethylene binding site (Serek *et al*., 1994). 1-MCP prevented the adverse effects of ethylene in ivy geraniums (Cameron and Reid, 2000) and *Pelargonium* cuttings (Serek *et al*., 1998). Therefore, the rapid decline in quality, which is observed in *Pelargonium* cuttings, was attributed to ethylene (Arteca *et al*., 1996; Serek *et al*., 1998).

Ethylene biosynthesis in carnation flowers is autocatalytically triggered by ethylene (Mor and Reid, 1981). However, in tobacco vegetative tissues such as leaves, ethylene production is auto-inhibited via a negative feedback control mechanisms (Philosoph-Hadas *et al*., 1985). Also, ethylene interacts with ABA during the senescence of oat leaf segments. ABA appears to be the initiating agent, whereas ethylene appears to exert its effect at a later stage (Gepstein

and Thimann, 1981). The role of ethylene in leaf senescence has been investigated genetically using the ethylene-insensitive *Arabidopsis* mutant *etr1-1* (Grbic and Bleecker, 1995) and transgenic tomato plants, whose ethylene synthesis was blocked by an *ACO* antisense gene (John *et al*., 1995). Blocking ethylene biosynthesis in these plants delayed senescence, suggesting ethylene action may be involved in leaf yellowing (Picton *et al*., 1993; John *et al.,* 1995). However, once senescence was initiated, the regulation of SAGs did not differ much from *Arabidopsis* wild-type plants (Grbic and Bleecker, 1995). These results suggest an age-dependent senescence program that does not involve the ethylene-dependent pathway, which is sufficient and necessary for leaf senescence (Nam, 1997).

Ethylene plays a role in adventitious rooting but the literature is conflicting. Recently, Kadner and Druege (2004) demonstrated that ethylene treatment of zonal *Pelargonium* 'Mitzou' cuttings increased root formation. Additionally, ethylene promoted rooting in mung bean cuttings (Robbins *et al*., 1985). Generally, ethylene affects various stages of rooting process differently. Small amounts of ethylene stimulate root initiation and thus the number of roots formed, but it inhibits root emergence and elongation (Riov and Yang, 1989). Liu *et al.* (1990) concluded that wound-induced increase in ethylene seen within 3 h of excision of Sunflower cuttings was the key stimulatory factor in the induction of root primordia.

2.1.3 Abscisic acid (ABA)

Apart from ethylene, other plant hormones like ABA and cytokinins are also involved in leaf senescence and rooting. ABA is synthesised in roots and mature leaves while seeds are also rich in ABA, which may be imported from the leaves or synthesised *in situ* (Rivier *et al.,* 1977). ABA is transported in both xylem and phloem, thus it has been detected in every major organ or tissue from the root cap to apical bud (Milborrow, 1984). Applying ABA at very low concentrations to roots of geranium cuttings consistently promoted a decrease in photosynthesis, transpiration and relative growth rate (Arteca *et al.,* 1985). Other reports showed that exogenously applied ABA accelerated leaf senescence (Kaiser *et al*., 1985; Smart, 1994). However, some reports may be related to stresses that are imposed and thus induce leaf senescence (Becker and Apel, 1993). Also, in many plant species, ABA accelerates senescence in both attached leaves and leaf segments (Kaiser *et al*., 1985). It also, induces premature appearance of proteinase activity in barley leaves (Quiles *et al*., 1995) with a pattern on activity gels similar to those observed in naturally senescing leaves. In contrast, the antisense suppression

of a phospholipase essential for ABA-stimulated senescence in *Arabidopsis* leaves had no effect on the normal course of senescence (Fan *et al*., 1997), implying senescence of *Arabidopsis* leaves are not influenced by ABA.

Both ABA and ethylene enhance senescence and are thought to interact in that ABA stimulates ethylene production in a number of species (Riov *et al*., 1990; Müller *et al*., 1999). However, the mechanisms of ABA-induced ethylene production are not clear. Ethylene production was restored to normal levels by exogenously applied ABA in *flacca* mutant of tomato (Tal *et al*., 1979). However, it was uncertain whether ethylene production was a direct result of ABA deficiency or indirect effect of water stress. Also, ABA stimulated ethylene production in citrus leaf and tomato fruit tissues directly via enhancement of ACC synthesis (Riov *et al*., 1990).

Leaf yellowing and abscission in miniature Roses was promoted by spraying ABA. However, it was unclear whether this was a direct ABA effect or was via ethylene, since no ethylene was detected in leaves (Müller *et al*., 1999). Also, ABA promoted senescence of detached rice leaves but inhibited water stress and ethylene production (Tsai *et al*., 1996). Also, treating detached rice leaf segments with inhibitors of ethylene action inhibited ABA-induced senescence (Tsai *et al*., 1996). However, 1-MCP did not reduce ABA-induced flower drop in miniature roses, suggesting it is not mediated via ethylene (Müller *et al*., 1999). Additionally, Zacarias and Reid (1990) showed that ethylene accelerated chlorophyll loss from wild-type *Arabidopsis* leaf discs but had no effect on yellowing of mutant discs. Contrary, ABA treatment stimulated chlorophyll loss in both *Arabidopsis* wild type and mutant discs. Zacarias and Reid (1990) concluded that although ABA stimulated ethylene production in *Arabidopsis* ethylene-insensitive mutant, its effects on leaf senescence were not mediated by ethylene.

ABA is known to inhibit root formation in vegetative cuttings (Kracke *et al*., 1981; Kelen and Ozkan, 2003). However, ABA does not always play a remarkable role in rooting process (Kracke *et al*., 1981). ABA reduced transpiration and maintained satisfactory water balance in geranium cuttings. This is not only useful in shipment and storage (Arteca *et al*., 1985), but it is also important for the success of rooting of cuttings (Loach, 1988). However, difficult to root grapevine rootstock cuttings contained higher ABA levels than easy-to-root cuttings (Kelen and Ozkan, 2003; Kracke *et al*., 1981). Also, endogenous ABA decreased during rooting process (Kracke and Cristoferi, 1983), implying a negative correlation between ABA and rooting rate (Kelen and Ozkan, 2003). Moreover, ABA accumulation plays a role in root

growth maintenance and/ or inhibition of shoot growth under water stress and depends on the response of a plant organ to ethylene (Saab *et al*., 1990). Also, roots maintain growth under dry soil conditions when supplied with high amounts of ABA. This is a further evidence for ABA interaction with ethylene in controlling root and shoots growth.

2.1.4 Dark-storage

Dark-induced senescence occurs in both old and young leaves, while natural senescence occurs in aged leaves "source", which enable supply of assimilates to young leaves and flowers "sink" (Weaver *et al.,* 1998). Dark-induced senescence is characterised by a chlorophyll loss in *Pelargonium* cuttings (Arteca *et al*., 1996; Serek *et al*., 1998), *Alstroemeria* cut flowers (Mutui *et al*., 2001, 2003) and both a decline in carbohydrates and gibberellins (GA_3) levels in *Alstroemeria* cut flowers (Jordi *et al*., 1994). Kadner and Druege (2004) found storage of *Pelargonium* 'Isabell' at 20^{o}C promoted leaf chlorosis and decay during rooting period and led to a higher percentage of decayed cuttings. Moreover, high storage temperatures lead to high metabolic processes that deplete carbohydrate and other reserves compounds in *Pelargonium* cuttings (Behrens, 1988; Arteca *et al*., 1996). Carbohydrates are necessary, not only for providing energy, carbon skeletons and osmotic potential, but also for interacting with plant signalling hormones such as cytokinins and ABA, which play a role in leaf senescence (Smart, 1994). Furthermore, difficult-to-root grapevine cuttings were unable to utilize their starch reserves, because they had low endogenous IAA levels (Kracke *et al*., 1981).

Stresses, such as mechanical wounding, drought or chemicals like auxins, heavy metals and other pollutants induces ethylene production in plant species (Abeles *et al*., 1992). Also, ethylene is involved in storage and transport-induced leaf senescence of *Pelargonium* cuttings (Purer and Mayak, 1989). Pre and post storage-induced leaf senescence was reduced by application of silver nitrate and silver thiosulphate (STS) (Carrow and Bahnemann, 1980; Paton and Schwabe, 1987; Purer and Mayak, 1989), suggesting involvement of ethylene. Also, 1-methylcyclopropene (1-MCP) retarded storage-induced leaf yellowing in *Pelargonium* cuttings (Serek *et al*., 1998). Additionally, 1-MCP increased ethylene evolution and partially reduced leaf senescence in *Pelargonium* cuttings during rooting period, after they were stored for 48 h at 20^{o}C (Kadner and Druege, 2004). However, STS and silver nitrate caused severe post storage injury and decay in *Pelargonium* 'Isabell' cuttings (Kadner

and Druege, 2004) during rooting that was similar to the one observed in *Epipremnum* cuttings (Müller *et al.,* 1997).

Successful storage of unrooted cuttings depends on storage conditions, state of cuttings and species (Behrens, 1988). Within the storage unit, it is best to maintain nearly 100% RH and maintain low temperature, as the hardiness of a given species can tolerate. This minimises dry matter loss and pathogen infestations (Behrens, 1988), which result in chlorosis, wilting and leaf abscission (Kirk *et al*., 1986). Therefore, the goal of post-harvest storage is to keep plant material alive and fresh for a long time by minimising stress-inducing factors, as well as slowing down normal physiological processes initiated by harvesting. These include; degradation of chlorophyll, protein and starch, temporary accumulation of sugars and free amino acids, increase in respiration and ethylene production (Kirk *et al*., 1986). Low pressure conditions improved storability of geranium cuttings (Eisenberg *et al*., 1978). Moreover, storage of unrooted geranium cuttings (*Pelargonium x hortorum* L. H. Bailey) was improved by high humidity in polyethylene bags stored at 4^{o}C and low irradiance illumination (1-30 µ mol m^{-2} s^{-1}) (Paton and Schwabe, 1987). In contrast, prestorage application of antitranspirants was detrimental to cuttings, but soaking their bases in 2 to 5% sucrose for 24 h prior to storage improved rooting (Paton and Schwabe, 1987). Eisenberg *et al*. (1978) observed a decrease in quality and rooting ability of many ornamental cuttings with increase in storage time. Eisenberg *et al*. (1978) concluded that maintaining post harvest quality of ornamental cuttings will remain a basic line of research.

2.1.5 Cytokinins

Cytokinins are senescence retardants and are biosynthesised in the root meristems (Itai and Birnbaum, 1996). They are translocated in riboside form via xylem to shoots, where they exert major regulatory influence on growth, photosynthesis and timing of senescence (Itai and Birnbaum, 1996). Also, cytokinins are involved in cell division, respiration, inorganic and organic nutrient mobilisation, enhancing flowering and other plant growth processes (Halevy and Mayak, 1981; Thimann, 1987). The first evidence that cytokinins were able to inhibit leaf yellowing or prevent accelerated protein loss was observed in detached *Xanthium* leaves (Richmond and Lang, 1957). Additionally, Mothes and Engelbrecht (1961) demonstrated that if a small area of basal leaf is treated with kinetin solution, only that area remains green while the rest of plant turns yellow. Cytokinins delays leaf senescence by arresting degradation of

chlorophyll, proteins (Sacher, 1973; Mutui *et al*, 2003), and delaying the onset of rising respiration in excised oat leaves (Thimann, 1987).

Synthetic cytokinins are used extensively in postharvest stage of floricultural plants to delay leaf senescence, but their mechanisms of action are not fully understood (Thimann, 1987; Smart, 1994). However, Salunke *et al.* (1962) explained that the primary step in degradation of soluble type RNA, which is one of the most important characteristics of senescence, is thought to involve the loss of end adenine group. Thus supplements of BA in holding solutions should provide the necessary adenine to restore the soluble RNA molecule. Conversely, exogenous cytokinins delayed leaf senescence in many plant species including *Pelargonium* (Purer and Mayak, 1988). Kinetin eliminated decay of leaves and retarded chlorophyll degradation in darkened geranium leaf tissues (Steinitz *et al.*, 1987). Also, when kinetin was applied before storage, it prevented poststorage senescence, decay of leaves and shoot cuttings stored with or without root primordia (Carrow and Bahnemann, 1980; Steinitz *et al.*, 1987). Moreover, Steinitz *et al*. (1987) found all detrimental effects (leaf yellowing, reduction in rooting percentage and main root numbers) of storage were avoided by prestorage formation of root primordia. Steinitz *et al*. (1987) concluded formation of root primordia is a simple and efficient means of reducing storage or shipment dependent loss of leafy cuttings quality.

Cytokinins interact with other plant hormones in regulation of senescence. Cytokinins antagonise the action of ABA and delay senescence of oat leaf segments (Gepstein and Thimann, 1981). In water stressed plants, levels of cytokinins have been reported to decrease while the level of ABA increases. Consequently, the leaves quickly turned yellow (Aharoni *et al*., 1977). In contrast, Suttle (1984) reported enhanced endogenous ACC levels and ethylene evolution in mung bean hypocotyls segments following TDZ treatment. Suttle (1984) observed that high levels of TDZ induced leaf abscission in cotton via increased high ethylene production. Application of BA in holding solutions has been shown to reverse or inhibit leaf senescence leading to a reduction in leaf chlorosis in *Alstroemeria* (Hicklenton, 1991; Mutui *et al*., 2001) and Easter lilies (Han, 1997; Heins *et al*., 1996). On the other hand, leaf chlorosis was promoted by ethylene in zonal *Pelargonium* (Purer and Mayak, 1988). Also, TDZ inhibited leaf yellowing and retarded chlorophyll degradation in cut tulips (Ferrante *et al*., 2003), but had little effect on the vase life of cut *Eucalyptus parvifolia* (Ferrante *et al*., 2002b).

Additionally, TDZ promoted lateral shoot elongation in cut chrysanthemum 'Regan giallo' (Ferrante *et al.,* 2003).

Histidine kinase (AHK4) is suggested to serve as a primary receptor that directly binds a variety of natural and synthetic cytokinins in *Arabidopsis*. These include aminopurines, such as isopentenyl-adenine or BA, and diphenylurea derivatives like TDZ; but not riboside derivatives (Yamada *et al*., 2001; Inoue *et al.* 2001). However, it is unclear whether TDZ acts to invoke cytokinin responses by interacting directly with cytokinin receptors in the leaves (Christianson and Hornbuckle, 1999), or indirectly by stimulating conversion of cytokinin nucleotides to their biologically active ribonucleosides (Capelle *et al*., 1983), or by inducing accumulation of endogenous adenine-based cytokinins (Thomas and Katterman, 1986), which could be due to inhibition of cytokinin oxidase (Hare and Van Staden, 1994). Ferrante *et al.* (2002a) concluded that the effectiveness of TDZ may result from a combination of these mechanisms. Also, transgenic *Arabidopsis* plants with a isopentenyl transferase (*ipt*) gene inserted that increases cytokinin levels showed a delay in onset of senescence and cytokinin activity increased in leaf tissue, when growing points were surgically removed (Smart, 1994). Regulation of senescence by naturally occurring cytokinins in *Phaseolus vulgaris* leaves, was attributed to their ability to delay expression of senescence associated receptor-like kinase (SARK), a senescence associated gene (SAG), which is exclusively expressed during senescence (Hajouj *et al*., 2000). SARK regulates initiation and / or the rate of progress of senescence syndrome at transcriptional level in aging leaves of *Phaseolus vulgaris* (Hajouj *et al*., 2000).

Endogenous cytokinins at normal physiological concentrations can either promote or inhibit root initiation and development, depending on their concentration and the plant species concerned (Fries, 1960; Bollmark *et al*., 1988). Conversely, both endogenous and synthetic kinetins stimulated dry weight and elongation of roots in lupin seedlings, but high kinetin levels strongly inhibited root elongation (Fries, 1960), wheat, flax and cucumber seedlings (Stendil, 1982). Moreover, when kinetin was in contact with roots for more than 2 days, it diminished the growth of roots and entire plant, but when applied to roots at very low levels, kinetin stimulated photosynthesis and plant growth (Dong and Arteca, 1982).

Exogenous cytokinins in rooting solutions of cuttings caused a strong inhibition of root formation and growth (Steinitz *et al.*, 1987; Bollmark *et al*., 1988; Ferrante *et al.,* 2003). Also,

a low level of endogenous cytokinins was found in pea cuttings during adventitious root formation (Bollmark *et al*., 1988). Applying CPPU, a synthetic cytokinin to apex of pea plants doubled the concentration of IAA, increased ethylene evolution at the base of cuttings and reduced root formation (Koukourikou-Petridou and Bangerth, 1997). Additionally, it was reported that cuttings of species with high natural cytokinin levels are more difficult to root than those with low cytokinin levels (Okoro and Grace, 1978). Moreover, internode and root elongation in *Arabidopsis* were both inhibited in transgenic plants expressing the cytokinin gene, isopentenyl transferase (*ipt*) and in cytokinin overproducing mutants (Cary *et al.*, 1995; Vogel *et al*., 1998). Cary *et al*. (1995) and Vogel *et al*. (1998) suggested it was possible that inhibition of hypocotyls and internode elongation induced by excess cytokinin, was due to production of ethylene and this inhibition represented another example of interdependence of hormonal regulatory pathways.

2.1.6 Auxin

Auxins promote cell elongation leading to plant growth. They may also be involved in delaying senescence, since aging is accompanied by a decrease in auxin levels, due to degradation by IAA oxidase (Halevy and Mayak, 1981). In contrast, Ahmad *et al*. (1987) found leafy cuttings of *Pisum sativum* treated with high concentrations of chlorinated auxins showed permanent epinasty, loss of apical growth and dominance, resulting in outgrowth of laterals from axillary buds. All these effects were ascribed to an increase in ethylene synthesis (Ahmad *et al*., 1987). Also, Sun and Bassuk (1993) reported that STS stimulated bud break and reduced root formation in rose cuttings. Sun and Bassuk (1993) assumed an increase in ethylene production after IBA treatment in plants, which inhibited bud break and stimulated rooting. Conversely, Sun and Bassuk (1993) concluded that use of ethylene action inhibitor, possibly stimulated bud break by blocking ethylene action.

The most pronounced effect of auxins is adventitious root formation. Rooting is a complex phenomenon, which involves numerous endogenous factors, among which auxin and ABA are believed to play a major role (Nordström and Eliasson, 1991). Auxins are regarded as universal inducers of rooting (Kelen and Ozkan, 2003). On the other hand, ABA is known to inhibit root formation (Kracke *et al*., 1981). IAA is the primary auxin found in plants but other indole compounds such as IBA and NAA have been isolated (Normanly *et al*., 1995). IAA is synthesised in young leaves (Feldman, 1980) and has diverse physiological roles in

plant growth such as cellular elongation, phototropism, geotropism, apical dominance, ethylene production and fruit development (Jarvis, 1986). Also, auxins are required for adventitious roots initiation on stems and division of first root initial cells are dependent upon them (Strömquist and Hansen, 1980).

There is a positive correlation between endogenous IAA levels in grapevine cuttings and number of roots produced (Kelen and Ozkan, 2003). Consequently, the lowest IAA levels are found in dormant and swollen bud stages (Kelen and Ozkan, 2003). Moreover, Gaspar *et al.* (1994) found the initiating phase of rooting (cell division and differentiation) was characterised by a decrease in free auxin levels to a minimum while the expressive phase of rooting (growth and emergence of root primordial) was associated with an increase in free auxin levels. This was in agreement with Koukourikou-Petridou and Bangerth (1997). After root initiation, IAA decreased either by degradation or conjugation but this had little effect on rooting, since root elongation requires little amounts of IAA (Jarvis, 1986; Kracke and Cristoferi, 1983).

Several stages of root formation exhibit extreme sensitivity to exogenous auxin and are correlated to endogenous auxin concentration shifts (King *et al*., 1995). Studies with a recessive *Arabidopsis* nuclear mutant, rooty (rty) showed that auxin is highly involved in root proliferation and restriction of shoot growth (King *et al*., 1995). Also, applying IBA to grapevine cuttings (Kracke and Cristoferi, 1983) or NAA (Koukourikou-Petridou and Bangerth, 1997) to pea stem cuttings promoted rooting. Therefore, it was suggested that root stimulating effects of IAA or IBA are closely associated with induction of ACC and ethylene biosynthesis (Riov and Yang, 1989; Robbins *et al*., 1985). This was because inhibitors of ethylene biosynthesis (AVG) and perception (STS and 1-MCP) have been shown to reduce root numbers in mung bean cuttings (Jusaitis, 1986; Robbins *et al*., 1985) and *Pelargonium* (Serek *et al*., 1998). Moreover, auxins have the ability to stimulate ethylene production in vegetative tissues of many plants (Abeles *et al*., 1992).

The objective of this study was to investigate the possible use of TDZ for improving the post harvest quality of *Pelargonium* cuttings against transport-associated stress and to elucidate how it modulates leaf senescence. Such stress involves short-term dark storage and ABA and ethylene as stress-response agents. IBA treatment was tested to alleviate a TDZ rooting inhibition effect in order to enhance the overall performance of the cuttings.

2.2 Materials and Methods

Plant material

Cuttings of five new *Pelargonium zonale* hybrid cultivars ('Fire', 'Ganymed', 'Greco', 'Katinka' and 'Surfing') considered to be susceptible to leaf yellowing were obtained from stock plants grown by a commercial breeder (Selecta Klemm GmbH & Co., KG, Stuttgart, Germany). They were rooted in commercially produced soil (Einheitserde, Werkverband E.V., Germany) and re-potted into 14 cm diameter pots 4 weeks later. They were subsequently grown in a greenhouse at the University of Hannover under the following conditions: 22°C day / 20°C night temperatures with 16 h supplementary irradiance of 100 µmol m^{-2} s^{-1} from SON-T lamps (Osram, 400W, Philips, Holland) between October-December to produce stock plants to provide experimental material. An automatic fertigation system was used to apply 0.75 % Wuxal^R Super fertiliser [8% (w/w) N, 8% (w/w) P_2O_5, 6% (w/w) K_2O, 0.01% (w/w) B, 0.007% (w/w) Cu, 0.015% (w/w) Fe, 0.013% (w/w) Mn, 0.001% (w/w) Mo, 0.005% (w/w) Zn; Wilhelm Haug GmbH & Co. KG, Ammerbuch-Pfäffingen, Germany] solution to the plants 1-3 times per week depending on the prevailing weather conditions.

Harvesting and evaluation of cuttings

Terminal cuttings were harvested with sterilised knife after 19 weeks growth, leaving the first two leaves of the axillary shoot on the stock-plant. The cuttings were 6 cm in length at most, and had 4 leaves of which at least one was fully developed. After harvesting, cuttings were transferred immediately to an interior environment room (IE) kept at 21°C $\pm$ 1°C, 60% RH and continuous light from cool-white fluorescent tubes (20 µmol m^{-2} s^{-1}). They were exposed to various treatments as described below. The air in the IE was ethylene-free. Evaluation of the cuttings was done initially, and at 2 and 4 days after treatment application. Leaf samples were taken from the cuttings for documentation by photography, colour and total chlorophyll content determination before they were rooted under hydroponics in greenhouses to evaluate their rooting potential.

Ethylene sensitivity

Cuttings were horizontally placed in sealed 54 l glass chambers. Ethylene gas was injected with a hypodermic syringe to give 0, 0.5, 1 and 2 µl l^{-1}. This procedure was repeated daily after 1h ventilation of the glass chambers until termination of the experiment. Ethylene concentrations inside the chambers were monitored using a Perkin-Elmer portable digital gas chromatograph (GC Voyager FFKG312, Canada) equipped with a photoionisation detector. The carrier gas was N_2 at 40 ml min^{-1}, the injection pressure was 69 kPa, the oven temperature was 60^{o}C and the column temperature was 60^{o}C. Control cuttings were kept sealed in an identical glass chambers, but without ethylene.

Thidiazuron (TDZ) treatment

TDZ (Sigma-Aldrich GmBH, Germany) was dissolved in 1M KOH to prepare stock solutions. Deionised water containing 0.2% (v/v) Tween 20 (Duchefa, Haarlem, The Netherlands) as wetting agent was used to prepare 5, 10 and 20 µM TDZ solutions. The foliage on cuttings was completely immersed in TDZ solutions for 1 min. Care was taken that no solution reached the stem base. Control cuttings were immersed in deionised water containing 0.2% (v/v) Tween 20. After treatment, the cuttings were laid on absorbent paper to dry for 30 minutes.

Abscisic acid (ABA) treatment

ABA (Precision Biochemicals, Germany) was dissolved in 1M KOH to prepare stock solutions. Deionised water containing 0.2% (v/v) Tween 20 (Duchefa, Haarlem, The Netherlands) as wetting agent was used to prepare 25, 50 and 100 µM ABA solutions. The foliage on cuttings was completely immersed in ABA solutions for 1 min. Care was taken that no solution reached the stem base. Control cuttings were immersed in deionised water containing 0.2% (v/v) Tween 20. After treatment, the cuttings were laid on absorbent paper to dry for 30 min.

Dark storage

Cuttings were placed in polyethylene bags and the top of the bag was tightly sealed with a band. They were then packed randomly into boxes and stored at 21^{o}C $\pm$ 1^{o}C in darkness for 4 days to simulate transport conditions.

Indole-3-butyric acid (IBA) treatment

IBA (Duchefa, Haarlem, The Netherlands) was dissolved in 1M KOH and deionised water was added to prepare a 500 mg l^{-1} IBA stock solutions. Aliquots of IBA stock solution were dissolved at the beginning of the experiment into 10 l nutrient solutions (as described below) to make 4, 8 and 12 µl l^{-1} IBA solutions. Control cuttings were placed in nutrient solutions without IBA.

Colour measurement

Representative colour measurements were performed in triplicate on the surface of individual leaves using a Minolta Chroma Meter (Model CR-300, Minolta, Osaka, Japan). This tri-stimulus colour analyser consists of a head with an 8 mm diameter measuring area, a diffuse illumination and a 0^{o} viewing angle. It was initially calibrated with a white tile and checked between measurements. The three measurements were done on the left, right and centre of each leaf blade, respectively, at the start of the experiment. In addition to the initial measurements, they were made at 2 and 4 days after TDZ, ethylene or ABA treatments. For dark storage treatment, measurements were taken initially and after 4 days. The three parameters of brightness (L^{*}), red-to-green scale (a^{*}) and yellow-to-blue scale (b^{*}) were recorded. Chroma and Hue were calculated using the formulae:

$$\text{Chroma} = (a^2 + b^2)^{0.5}$$

$$\text{Hue} = \arctan (b/a)$$

High chroma and low hue values indicate that the leaves turn yellow due to onset of senescence. Low chroma and high hue values indicate that the leaves remain green. Leaf chroma refers to the saturation of the colour, which is how intense, pure or vivid it is while leaf hue refers to visual sensation or tone of the colour.

Chlorophyll determination

Leaves without petioles were weighed (fresh weight). Then, three 8 mm diameter discs were excised from left, centre and right, respectively, of each leaf blade using a cork borer. Chlorophyll content was analyzed according to Lichtenthaler (1987). Extraction was in 80% (v/v) ethanol at 75^{o}C for 10 min. Absorption was measured using a SmartSpec^{TM} 3000 Spectrophotometer (Biorad, California) at 647, 664 and 700 nm. Chlorophyll content was calculated using the equation:

$$\text{Chlorophyll}_{a+b} = 5.24(A_{664}-A_{700}) + (A_{647}-A_{700})22$$

Where, A is absorbance at 647 nm, 664 nm and 700 nm. The results were expressed as mg chlorophyll cm^{-2} of leaf area.

Rooting of cuttings

Rooting was done in a greenhouse under the following conditions: 24^{o}C$\pm$ 1^{o}C temperature inside the rooting chamber, 97$\pm$1% RH, and supplementary irradiance from SON-T lamps (Osram, 400W, Philips, Holland) at 60 µmol m^{-2} s^{-1}. Eight cuttings for each treatment level were placed in grey Styropor^{TM} plates and floated on nutrient solution in 10 l containers (Fig. 1). Containers were covered with non-transparent white polyethylene which, together with the grey Styropor^{TM} plates, substantially reduced the amount of light reaching the base of the cuttings. The nutrient solution was continuously aerated to prevent oxygen depletion. The composition of the nutrient solution was as follows (mg salt l^{-1}): NH_4NO_3, 12; K_2PO_4, 162.8; $MgSO_4$, 71.2; KNO_3, 174; $Mg(NO_3)_2$, 487; FeEDTA, 12; MnSO4, 1.9; $ZnSO_4$, 2.4; $CuSO_4$, 0.36; H_3BO_3, 1.9; $NaMoO_4$, 0.16; NaCl, 25.5; and, $Ca(NO_3)_2$, 861. Propagation vessels were placed under a white polyethylene tent to increase humidity. The rooting period was 4 weeks.

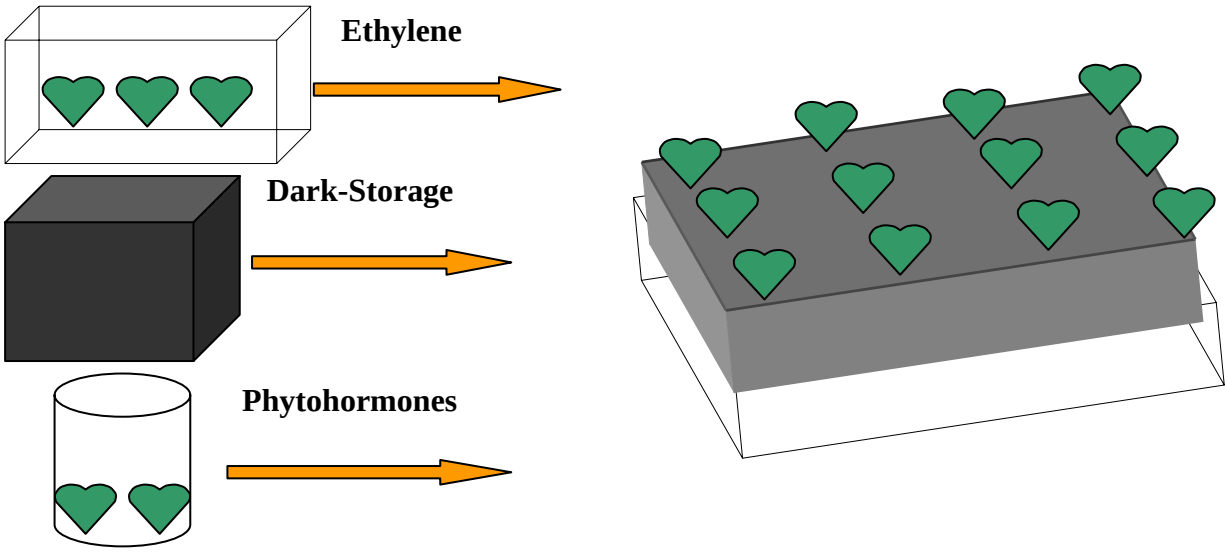

Fig. 1. Schematic presentation of application of treatments and rooting of *Pelargonium* cuttings by hydroponics.

Rooting parameters

The percentage (%) of rooted cuttings was determined by counting the number of rooted cuttings against the initial number that was placed in the rooting solution. The number and length of roots per cutting were also determined. The roots were weighed using a balance ELE (Sartorious GmbH, Göttigen, Germany) to obtain fresh weight. The root samples were then wrapped in aluminium foil, oven dried at 66°C for 72 hr to constant weight using incubator (Memmert GmbH, Schwabach, Germany), cooled in a desiccator for 30 min and re-weighed for dry weight. Root water content was determined by subtracting root dry weights from their corresponding fresh weights.

Experimental design and statistics

The experiments were conducted in a completely randomised design using two replications per treatment of four sample cuttings per replication. Data were subjected to a single factor analysis of variance (ANOVA) using the general linear models (Proc GLM) of the Statistical Analysis System (SAS, 2002) program package. Multiple comparisons among treatment means was done using the Least Significant Difference (LSD) or Student's *t* test at P = 0.05.

2.3 Results

Effects on leaf colour

Storing *Pelargonium* cuttings in the dark for 4 days and/ or treating them with 2 µl l^{-1} ethylene or 100 µM ABA hastened the onset of visible leaf yellowing in 'Fire', 'Ganymed', 'Greco', 'Katinka' and 'Surfing'. This was documented by photography for 'Katinka' which was representative of the other 4 cultivars (Fig. 2A, B, C). In contrast, treatment with 5 µM TDZ retarded leaf yellowing in 'Katinka' (Fig. 2D).

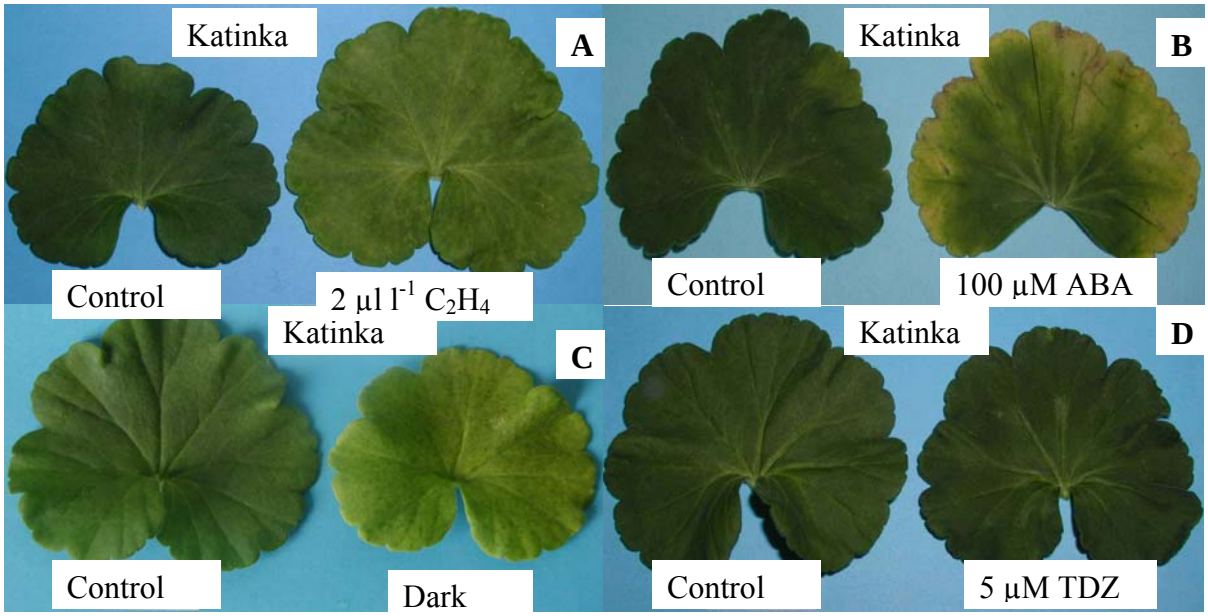

Fig. 2. Appearance of representative *Pelargonium* cv 'Katinka' leaves after continued exposure to (A) 2 µl l^{-1} ethylene for 4 days, (B) application of 100 µM ABA for 4 days, (C) dark storage for 4 days and (D) application of 10 µM TDZ for 4 days.

Treating *Pelargonium* cuttings with 2 µl l^{-1} ethylene increased leaf chroma values throughout the study period in all five cultivars investigated (Fig. 3A, B). However, 0.5 µl l^{-1} ethylene had no effect on leaf chroma values and was not different from 1 µl l^{-1} ethylene for 'Ganymed', 'Katinka' and 'Surfing' after both 2 and 4 days (Fig. 3A, B).

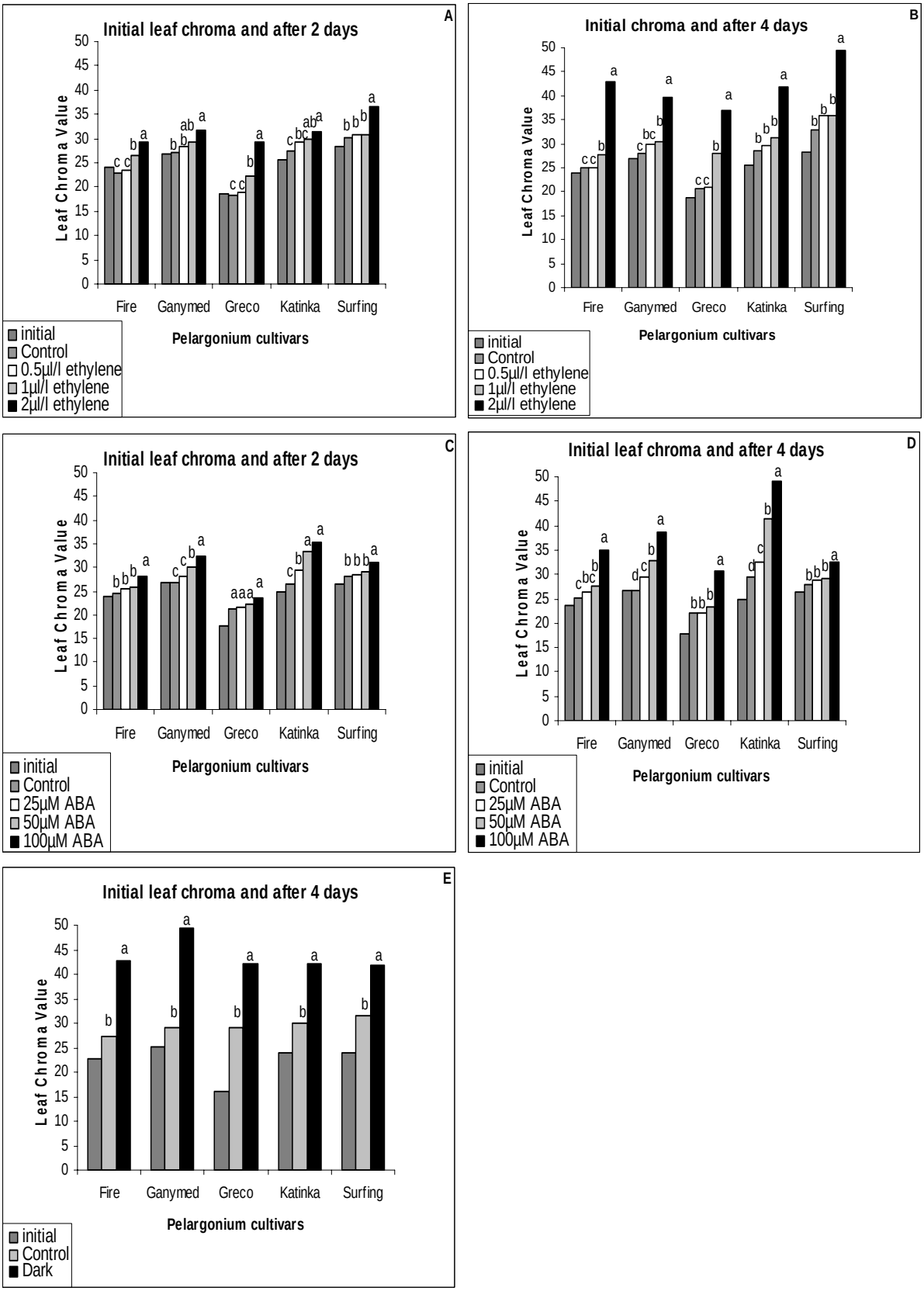

Fig. 3. Effects of continuous exposure to (A,B) 0.5, 1 and 2 µl l^{-1} ethylene for 4 days, (C,D) application of ABA at 25, 50 or 100 µM for 4 days and (E) dark storage for 4 days on leaf chroma of five *Pelargonium* cultivars. Means separated by LSD (P=0.05), means followed by the same letter(s) within the cultivar are not significantly different.

Applying ABA at 100 µM increased leaf chroma throughout the study period in all cultivars investigated except 'Greco' where the difference was not apparent after 2 days (Fig. 3C, D). Contrary, 25 µM ABA had no effect on leaf chroma except in 'Katinka' for all days of observation (Fig. 3C, D) and 'Ganymed' after four days (Fig. 3D). Furthermore, application of 50 µM ABA increased leaf chroma for 'Ganymed' and 'Katinka' during the experimental period (Fig. 3C, D and 'Fire' after four days (Fig. 3D).

Storing cuttings in the dark for 4 days increased leaf chroma for all five cultivars studied (Fig. 3E). Treating the cuttings with 20 µM TDZ decreased leaf chroma after 2 or 4 days in all five cultivars (Fig. 4A, B). Furthermore, there was no difference among TDZ levels except in 'Fire' where 5 µM was less effective. Overall, 20 µM TDZ was the most effective level especially in 'Surfing' in preventing onset of yellowing in the five investigated cultivars (Fig. 4A, B).

Leaf hue (computed using –a* values) decreased for all cultivars after 2 and 4 days of continued exposure to 2 µl l^{-1} ethylene (Fig. 5A, B), except in 'Katinka' first after 4 days (Fig. 5B). Just like in leaf chroma, 0.5 µl l^{-1} ethylene had no effect on leaf hue values in all cultivars studied except in 'Surfing', when it first decreased leaf hue after 4 days (Fig. 5B). Moreover, 0.5 µl l^{-1} ethylene was not significantly different from 1 µl l^{-1} ethylene except in 'Fire' after 2 days and 'Greco' in both days of evaluation (Fig. 5A, B). Treating the foliage of cuttings with 100 µM ABA decreased leaf hue in all five cultivars for all the days of observation (Fig. 5C, D). Additionally, 25 µM ABA had no effect on leaf hue values in all cultivars. Furthermore, 50 µM ABA was only different from 25 µM ABA for 'Fire' and 'Ganymed' after 2 days and likewise for 'Katinka' and 'Ganymed' after 4 days (Fig. 5C, D).

Dark storage of the cuttings for 4 days decreased leaf hue values for all five cultivars investigated (Fig. 5E). In contrast, applying 20 µM TDZ to the cuttings foliage, consistently increased leaf hue values throughout the experimental period (Fig. 4C, D), except for 'Fire' after 2 days when it had no effect (Fig. 4C). Two days after TDZ treatment, there was no significant difference between 5 µM and 10 µM TDZ (Fig. 4C). Moreover, there was no discernable difference among TDZ levels after 4 days, except in 'Fire' where 5 µM was less effective (Fig 4D). Furthermore, 20 µM TDZ was the most effective in preventing the onset of leaf yellowing in all cultivars.

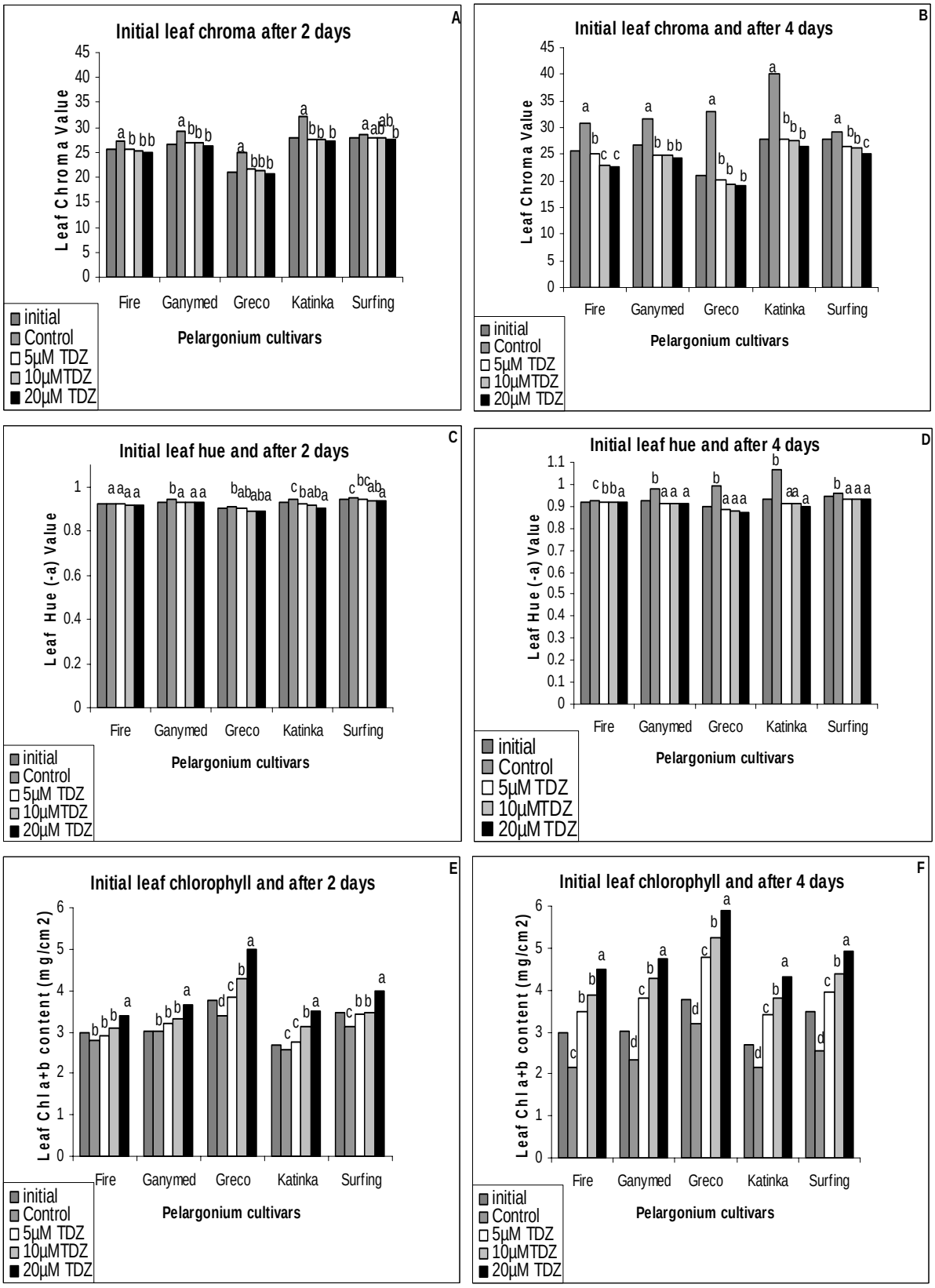

Fig. 4. Effects of application of TDZ at 5, 10 and 20 µM for 4 days on (A,B) leaf chroma, (C,D) leaf hue and (E,F) total leaf chlorophyll content of five *Pelargonium* cultivars. Means separated by LSD (P=0.05), means followed by the same letter(s) within the cultivar are not significantly different.

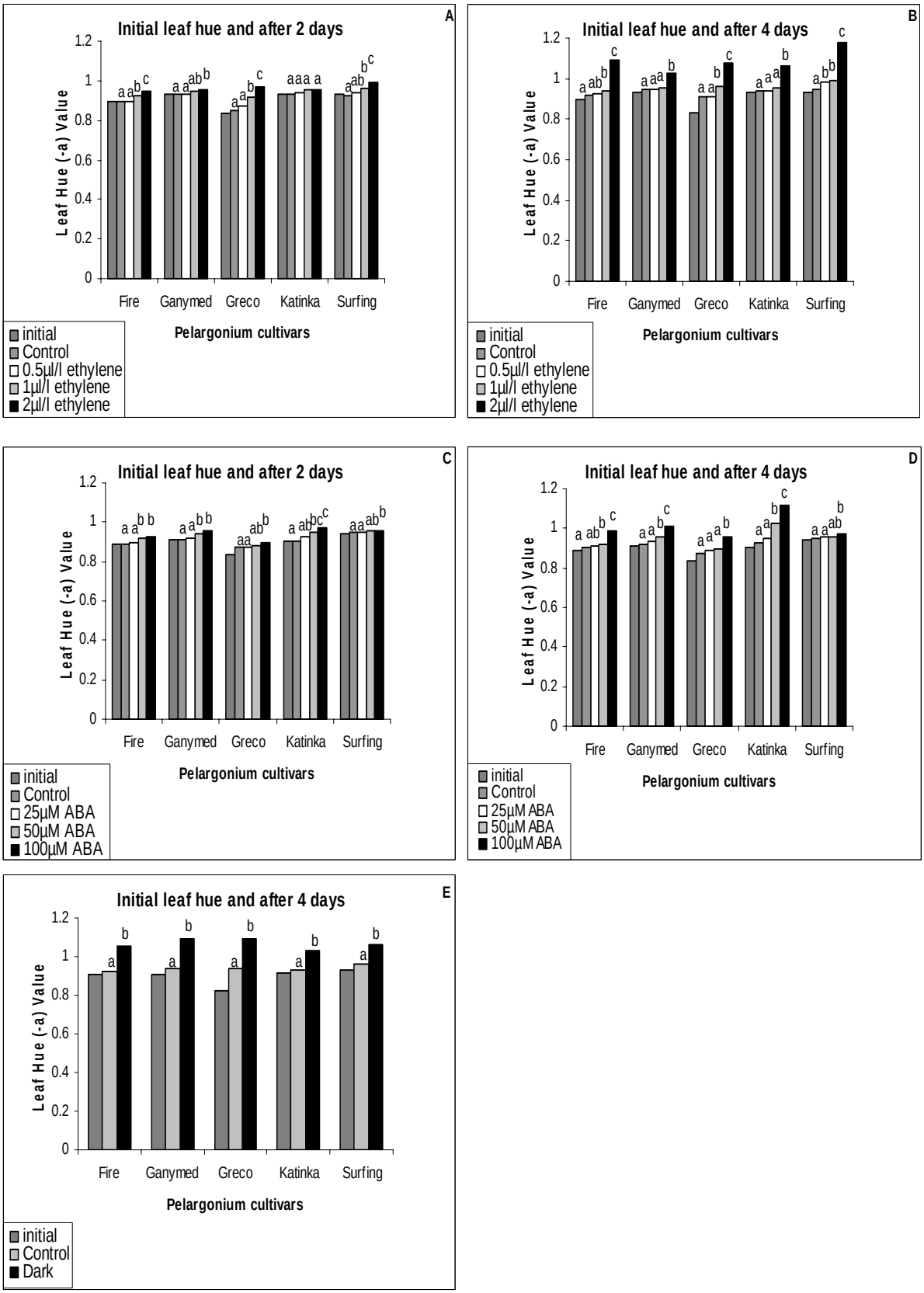

Fig. 5. Effects of continued exposure to (A,B) 0.5, 1 and 2 µl l^{-1} ethylene for 4 days, (C,D) application of ABA at 25, 50 or 100 µM for 4 days and (E) dark storage for 4 days on leaf hue of five *Pelargonium* cultivars. Means separated by LSD (P=0.05), means followed by the same letter(s) within the cultivar are not significantly different.

Effects on leaf chlorophyll content

During the experimental period, all ethylene levels significantly decreased total leaf chlorophyll content compared to the untreated control in all five *Pelargonium* cultivars investigated (Fig. 6A, B). Additionally, ethylene levels were significantly different, with the highest level of 2 µl l^{-1} being the most effective at promoting chlorophyll degradation (Fig. 6A, B). Similarly, total chlorophyll content also decreased in leaves of all five cultivars after treatment with ABA (Fig. 6C, D). This effect was greatest in leaves treated with the highest concentration of 100 µM ABA. Likewise, all the levels of ABA were significantly different throughout the period of study. Moreover, storing *Pelargonium* cuttings in the dark for 4 days decreased total leaf chlorophyll in all cultivars studied (Fig. 6E).

Chlorophyll content of *Pelargonium* leaves treated for 4 days with the lowest TDZ concentration of 5 µM TDZ was higher than their initial content (Fig. 4F), while chlorophyll content declined in the untreated controls. Similarly, 5 µM TDZ increased leaf chlorophyll content in 'Greco' and 'Surfing' after 2 days but had no effect on other cultivars (Fig. 4E). Additionally, 5 µM TDZ was not different from 10 µM TDZ after 2 days in all cultivars except in 'Greco' and 'Katinka', where the latter level was more effective (Fig. 4E). Four days after TDZ application, all the TDZ levels were significantly different with respect to leaf chlorophyll content except in 'Fire' where 5 µM TDZ was not different from 10 µM (Fig. 4F). TDZ at 20 µM consistently increased leaf chlorophyll contents (Fig. 4E, F).

Effects on adventitious root formation

Due to genotypic variation among the cuttings, the control for each individual cultivar was taken as having 100% rooting. Ethylene at 2 µl l^{-1} induced root formation in 'Greco', 'Katinka' and 'Surfing'. This effect was expressed in increased rooting proportion, and the same trend was true for 'Fire' (Table 1). Additionally, when ethylene was applied at lower levels (0.5 µl l^{-1}), root induction was observed in 'Greco' and 'Surfing'. Moreover, 'Fire' and 'Ganymed' showed a similar trend, but the beneficial effect of 0.5 µl l^{-1} ethylene was not apparent in 'Katinka'. In contrast, ethylene treatment reduced the ability of cuttings to continue growth of regenerated roots. This was evidenced by reduced numbers of roots per cutting in all cultivars (Fig.7A; Table 1) but this was not apparent in 'Greco' (Table 1).

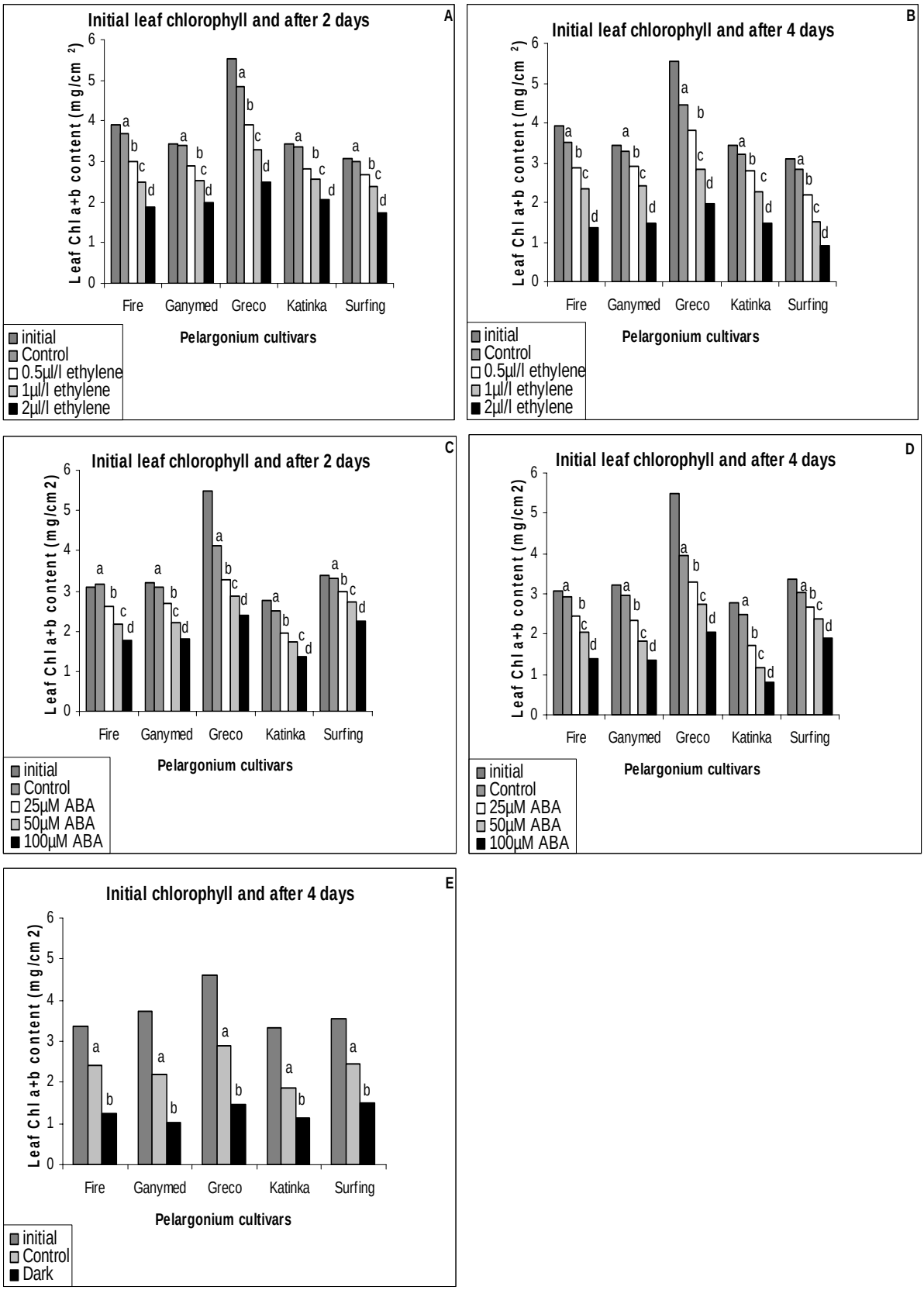

Fig. 6. Effects of continued exposure to (A,B) 0.5, 1 and 2 µl l^{-1} ethylene for 4 days, (C,D) application of ABA at 25, 50 or 100 µM for 4 days and (E) dark storage for 4 days on total leaf chlorophyll content of five *Pelargonium* cultivars. Means separated by LSD (P=0.05), means followed by the same letter(s) within the cultivar are not significantly different.

Likewise, ethylene reduced the length of roots in all investigated cultivars (Fig. 7A) except in 'Greco' and 'Surfing', where it had no effect. All ethylene levels had similar effect (Table 1). Furthermore, root fresh weight of 'Katinka' and 'Surfing' reduced after exposure to ethylene for 4 days (Table 1). The same trend was observed for the other three cultivars. In contrast, 0.5 µl l^{-1} ethylene increased the root dry matter (%) accumulation in 'Greco' and 'Surfing' (Table 1) and the same trend was true for 'Katinka'. Similarly, 0.5 and 1 µl l^{-1} ethylene reduced root water content (%) in 'Greco' and 'Surfing', respectively. Also, 2 µl l^{-1} ethylene exhibited a similar trend in 'Katinka' (Table 1).

Treating *Pelargonium* cuttings with both 50 µM and 100 µM ABA retarded root induction as expressed by rooting proportion in 'Ganymed' and 'Katinka' (Table 2). Also, 25 µM ABA had the same effect in 'Surfing' and 'Katinka'. Similarly, 50 µM ABA reduced the ability of cuttings to continue growth of roots. This was shown by reduced numbers of roots per cutting in all cultivars (Fig. 7B) except 'Ganymed', where this effect was not apparent (Table 2). All levels of ABA comparably reduced the lengths of roots per cutting in all cultivars investigated (Fig. 7B; Table 2). The fresh and dry weights of roots followed essentially the same pattern as the root lengths except in 'Surfing', where 50 µM ABA had no effect on root dry weights (Table 2). Moreover, ABA levels comparably reduced root water content and showed a similar pattern to that exhibited by root fresh weights (Table 2).

Storing *Pelargonium* cuttings for 4 days in the dark had no effect on the rooting proportion in all cultivars (Table 3). However, short-term dark storage reduced the number of roots per cutting by 50% in 'Katinka' (Fig. 7C; Table 3). Likewise, darkness reduced the lengths of induced roots in 'Surfing' and 'Katinka' (Fig. 7C; Table 3). The fresh weights, dry weights and water content of the roots followed essentially the same pattern as the root length in 'Greco' and 'Katinka' (Table 3).

TDZ severely inhibited root formation at the base of stems of the cuttings (Fig. 7D). This resulted in just 1-2% rooting of all cuttings, in five *Pelargonium* cultivars tested (data not presented). TDZ treatment also led to browning of the stem tissues after 8 days, followed by blackening 7 days later (Fig. 7D).

Fig. 7. Appearance of representative *Pelargonium* cv 'Katinka' roots after continued exposure to (A) 2 µl l^{-1} ethylene for 4 days, (B) application of 100 µM ABA for 4 days, (C) dark storage for 4 days, (D) application of 5 µM TDZ for 4 days, (E) application of 4 µl l^{-1} IBA in rooting solution and (F) pre-treatment with 5 µM TDZ for 4 days followed by application of 4 µl l^{-1} IBA in rooting solution for 28 days.

Table 1
Effect of continued exposure to 0.5, 1 and 2 µl l^{-1} ethylene for 4 days on root induction and growth in cuttings of five *Pelargonium* cultivars.

Cultivar	Treatment	Rooting (%)	Number of roots	Length of roots (cm)	Roots FW (mg)	Roots DW (%)	Roots WC (%)
'Fire'	Control	100a	**12.80a**	**155.05a**	323.80a	6.77a	93.23a
	0.5 µl l^{-1} C_2H_4	125a	**4.50b**	**56.03b**	243.80a	6.33a	93.67a
	1.0 µl l^{-1} C_2H_4	125a	**7.00b**	**114.13ab**	376.30a	7.99a	92.01a
	2.0 µl l^{-1} C_2H_4	125a	**5.80b**	**78.63ab**	341.30a	7.69a	92.31a
	LSD	ns	**4.96**	**90.40**	ns	ns	ns
'Ganymed'	Control	100a	**12.50a**	**178.08a**	497.50a	8.11a	91.89a
	0.5 µl l^{-1} C_2H_4	125a	**4.00b**	**68.78b**	256.30a	7.91a	92.09a
	1.0 µl l^{-1} C_2H_4	125a	**6.50ab**	**114.45ab**	350.00a	7.63a	92.37a
	2.0 µl l^{-1} C_2H_4	100a	**4.30b**	**79.48ab**	281.30a	8.74a	91.26a
	LSD	ns	**6.14**	**103.11**	ns	ns	ns
'Greco'	Control	**100b**	5.80a	79.83a	317.50a	**18.07b**	**94.40a**
	0.5 µl l^{-1} C_2H_4	**150a**	3.50a	62.00a	200.00a	**26.46a**	**91.79b**
	1.0 µl l^{-1} C_2H_4	**125ab**	4.00a	62.08a	125.00a	**13.79b**	**92.84ab**
	2.0 µl l^{-1} C_2H_4	**125ab**	6.50a	92.15a	177.50a	**14.44b**	**92.49b**
	LSD	**20.96**	ns	ns	ns	**4.31**	**1.78**
'Katinka'	Control	**100b**	**9.80a**	**209.15a**	**1482.50a**	40.7ab	94.19ab
	0.5 µl l^{-1} C_2H_4	**100b**	**1.00b**	**12.20b**	**263.50b**	64.05a	96.01a
	1.0 µl l^{-1} C_2H_4	**100b**	**3.50b**	**70.80b**	**422.50b**	41.65ab	95.32a
	2.0 µl l^{-1} C_2H_4	**150a**	**2.00b**	**35.40b**	**108.30b**	32.15b	87.89b
	t value	**2.18**	**2.57**	**2.57**	**2.57**	ns	ns
'Surfing'	Control	**100c**	**11.67a**	180.47a	**965.00a**	**34.68b**	**93.06a**
	0.5 µl l^{-1} C_2H_4	**133b**	**8.80ab**	139.48a	**987.50a**	**55.32a**	**92.11ab**
	1.0 µl l^{-1} C_2H_4	**133b**	**6.50b**	105.50a	**446.30b**	**38.99b**	**90.64b**
	2.0 µl l^{-1} C_2H_4	**200a**	**7.50ab**	146.53a	**600.00ab**	**35.91b**	**91.26ab**
	t value	**2.18**	**2.20**	ns	**2.20**	**2.20**	**2.20**

Means separated by LSD (P=0.05). For each cultivar, means followed by the same letter(s) within columns are not significantly different. N=8.

Table 2
Effect of application of 25, 50 and 100 µM ABA for 4 days on root induction and growth in cuttings of five *Pelargonium* cultivars.

Cultivar	Treatment	Rooting (%)	Number of roots	Length of roots (cm)	Roots FW (mg)	Roots DW (mg)	Roots WC (mg)
'Fire'	Control	100a	**6.60a**	**98.86a**	**871.30a**	**36.54a**	**834.80a**
	25 µM ABA	100a	**4.60b**	**62.30b**	**711.10ab**	**28.83ab**	**682.20ab**
	50 µM ABA	100a	**4.10b**	**54.01b**	**521.10b**	**24.29b**	**496.80b**
	100µM ABA	100a	**3.50b**	**48.38b**	**502.50b**	**26.49b**	**476.00b**
	LSD	ns	**1.26**	**23.39**	**342.61**	**9.96**	**334.49**
'Ganymed'	Control	**100a**	**6.30a**	**112.29a**	**1080.30a**	**46.21a**	**1034.10a**
	25 µM ABA	**100a**	**5.10a**	**77.19ab**	**748.00ab**	**35.88ab**	**712.20ab**
	50 µM ABA	**90b**	**5.30a**	**83.19ab**	**645.10ab**	**29.25ab**	**615.90ab**
	100 µM ABA	**90b**	**3.50b**	**52.63b**	**468.70b**	**20.00b**	**448.70b**
	LSD	**7.74**	**1.37**	**35.17**	**517.47**	**17.85**	**502.03**
'Greco'	Control	**100b**	**4.00a**	**53.98a**	**599.40a**	**23.71a**	**575.70a**
	25 µM ABA	**111a**	**3.80ab**	**53.18ab**	**377.50ab**	**19.12ab**	**358.30ab**
	50 µM ABA	**111a**	**2.80b**	**34.60b**	**269.50b**	**13.99b**	**255.50b**
	100 µM ABA	**100b**	**3.30ab**	**45.79ab**	**556.00ab**	**21.00ab**	**535.00ab**
	LSD	**7.74**	**1.10**	**19.23**	**322.74**	**8.32**	**316.25**
'Katinka'	Control	**100a**	**3.40a**	**52.48a**	**329.75a**	**16.34a**	**313.41a**
	25 µM ABA	**89b**	**2.30b**	**32.33b**	**194.50b**	**10.55b**	**183.95b**
	50 µM ABA	**89b**	**2.60b**	**38.20b**	**183.20b**	**11.03b**	**172.17b**
	100 µM ABA	**89b**	**2.10b**	**27.29b**	**135.04b**	**9.25b**	**125.79b**
	LSD	**5.47**	**0.73**	**13.48**	**85.60**	**5.17**	**83.25**
'Surfing'	Control	**100a**	**5.25a**	**67.13a**	**778.70a**	**32.21a**	**746.50a**
	25 µM ABA	**80b**	**3.25b**	**37.05b**	**279.80b**	**15.54b**	**264.30b**
	50 µM ABA	**100a**	**3.50b**	**42.48b**	**497.20b**	**32.46a**	**464.70b**
	100 µM ABA	**90ab**	**3.63b**	**42.83b**	**419.90b**	**17.97b**	**401.90b**
	LSD	**12.24**	**1.27**	**18.30**	**278.45**	**12.88**	**268.04**

Means separated by LSD (P=0.05). For each cultivar, means followed by the same letter(s) within columns are not significantly different. N=8.

Table 3

Effect of dark storage for 4 days on root induction and growth in cuttings of five *Pelargonium* cultivars.

Cultivar	Treatment	Rooting (%)	Number of roots	Length of roots (cm)	Roots FW (mg)	Roots DW (mg)	Roots WC (mg)
'Fire'	Control	100a	11.30a	280.63a	2072.90a	83.13a	1989.80a
	Stored	100a	7.50a	116.38a	928.40a	38.88a	889.50a
	LSD	ns	ns	ns	ns	ns	ns
'Ganymed'	Control	100a	6.80a	119.38a	924.80a	39.63a	885.10a
	Stored	100a	7.50a	102.75a	1264.10a	46.25a	1217.90a
	LSD	ns	ns	ns	ns	ns	ns
'Greco'	Control	100a	6.00a	102.00a	**775.60a**	**31.13a**	**744.50a**
	Stored	100a	4.30a	63.13a	**393.60b**	**16.63b**	**377.00b**
	LSD	ns	ns	ns	**382.32**	**14.47**	**368.69**
'Katinka'	Control	100a	**8.00a**	**178.13a**	**954.00a**	**48.00a**	**906.00a**
	Stored	83.34a	**4.00b**	**45.13b**	**368.10b**	**18.25b**	**349.90b**
	LSD	ns	**2.45**	**77.12**	**578.78**	**25.68**	**553.87**
'Surfing'	Control	100a	8.30a	**158.38a**	1019.40a	52.75a	966.60a
	Stored	120a	6.50a	**99.13b**	717.80a	30.88a	686.90a
	LSD	ns	ns	**59.11**	ns	ns	ns

Means separated by Least Significant Difference (P=0.05). For each cultivar, means followed by the same letter(s) within columns are not significantly different.

N=8.

Pelargonium cuttings were treated with a range of IBA concentrations dissolved in the rooting solutions to determine if exogenous auxin could overcome the root inhibitory effects of TDZ. Preliminary results showed that IBA could not overcome the root inhibitory effects of TDZ in 'Greco' and 'Surfing' (data not presented). Therefore, further investigations were conducted with 'Fire', 'Ganymed' and 'Katinka'. Treating cuttings with 4 µl l^{-1} IBA resulted in 100% root induction in 'Ganymed' and compared favourably to the control (Table 4). However, the proportion of rooted cuttings declined with higher (8 µl l^{-1} and 12 µl l^{-1}) IBA concentrations in the three cultivars tested (Table 4). Similarly, there was a reduction in the number of roots, and this was more pronounced when IBA was applied at 12 µl l^{-1} in 'Fire' and 'Katinka'. Also, 'Ganymed' exhibited the same trend. In contrast, this decline in the number of roots was not apparent at 4 µl l^{-1} and 8 µl l^{-1} IBA levels (Fig. 7E). Additionally, 4 µl l^{-1} and 8 µl l^{-1} IBA compared favourably with the control in all cultivars studied (Table 4). The lengths of the roots were not changed by application of 4 µl l^{-1} IBA in rooting solutions for 'Fire' and 'Katinka' (Fig. 7E, Table 5). However, 8 µl l^{-1} and 12 µl l^{-1} IBA retarded root growth as shown by reduced length of the roots in all cultivars. Fresh weights of roots followed the same pattern as root lengths. IBA at 4 µl l^{-1} resulted in a profound increase and reduction in root dry matter (%) accumulation and root water content (%), respectively, in all three cultivars (Table 4).

TDZ severely inhibited root formation, therefore control cuttings were treated with deionised water containing 0.2% (v/v) Tween 20. Based on IBA results (Table 4) cuttings were treated with 5 µM TDZ followed by application of 4, 8 or 12 µl l^{-1} IBA in the rooting solutions. Treating cuttings of 'Fire', 'Ganymed' and 'Katinka' with 5 µM TDZ followed by application of 4 µl l^{-1} IBA in rooting solutions at the beginning of experiment, restored their rooting abilities. This effect was evidenced by high rooting proportion in all cultivars (Table 5). Applying 4 µl l^{-1} IBA increased rooting proportions in 'Fire' and 'Katinka' and numbers of roots per cutting in 'Katinka'. However, IBA at 8 µl l^{-1} or 12 µl l^{-1} reduced root induction in all cultivars (Table 5). When placed in rooting solution containing 4 µl l^{-1} IBA, cuttings of 'Fire' and 'Ganymed' increased their root length. A similar trend was observed in 'Katinka' (Fig. 7F). Likewise 'Katinka' exhibited a similar pattern to root lengths with respect to root fresh weights (Table 5). Applying 8 µl l^{-1} IBA had a markedly increased dry weight (%) by 15% and reduced root water content (%) by 1% in 'Fire' (Table 5). Generally, high (8 and 12 µl l^{-1}) IBA levels were not different from each other for all cultivars, except rooting proportion in 'Fire' and root lengths in 'Ganymed' (Table 5).

Table 4

Effect of application of 4, 8 and 12 µl l^{-1} IBA in the rooting solution for 28 days on root induction and growth for five *Pelargonium* cultivars.

Cultivar	Treatment	Rooting (%)	Number of roots	Length of roots (cm)	Roots FW (mg)	Roots DW (%)	Roots WC (%)
'Fire'	Control	**100a**	**5.50a**	**52.00a**	**1263.60a**	**4.73b**	**95.27a**
	4 µl l^{-1} IBA	**82.80b**	**5.10ab**	**41.00ab**	**827.90ab**	**7.36a**	**92.63b**
	8 µl l^{-1} IBA	**71.40b**	**5.10ab**	**27.30b**	**913.00ab**	**4.84b**	**95.16a**
	12 µl l^{-1} IBA	**52c**	**3.80b**	**38.60ab**	**539.40b**	**4.65b**	**95.35a**
	t value	**2.07**	**2.07**	**2.07**	**2.07**	**2.07**	**2.07**
'Ganymed'	Control	**100a**	6.30a	**80.88a**	**1593.60a**	**3.93b**	**96.07a**
	4 µl l^{-1} IBA	**100a**	5.40a	**39.88b**	**755.40b**	**6.36a**	**93.64b**
	8 µl l^{-1} IBA	**94ab**	6.00a	**36.13b**	**1001.60ab**	**4.26b**	**95.74a**
	12 µl l^{-1} IBA	**89b**	4.80a	**46.00b**	**740.90b**	**4.38b**	**95.62a**
	t value	**2.05**	ns	**2.05**	**2.05**	**2.05**	**2.05**
'Katinka'	Control	**100a**	**5.10a**	**61.50a**	**727.10a**	**4.70b**	**95.29a**
	4 µl l^{-1} IBA	**71.40b**	**4.10ab**	**45.86ab**	**498.70ab**	**6.92a**	**93.07b**
	8 µl l^{-1} IBA	**71.40b**	**4.00ab**	**24.57b**	**479.40ab**	**4.36b**	**95.64a**
	12 µl l^{-1} IBA	**52c**	**2.80b**	**37.40ab**	**359.60b**	**4.25b**	**95.74a**
	t value	**2.07**	**2.07**	**2.07**	**2.07**	**2.07**	**2.07**

Means separated by Student's *t* test (P=0.05). For each cultivar, means followed by the same letter(s) within columns are not significantly different.

N=8.

Table 5

Effect of treating cuttings with 5 µM TDZ followed by application of 4, 8 and 12 µl l^{-1} IBA in rooting solution for 28 days on root induction and growth for five *Pelargonium* cultivars.

Cultivar	Treatment	Rooting (%)	Number of roots	Length of roots (cm)	Roots FW (mg)	Roots DW (%)	Roots WC (%)
'Fire'							
	DI Water	**100b**	**4.10ab**	**28.43bc**	**603.40ab**	**5.68b**	**94.32a**
	5 µM TDZ + 4 µl l^{-1} IBA	**140a**	**5.50a**	**49.25ab**	**889.60a**	**5.41b**	**94.59a**
	5 µM TDZ + 8 µl l^{-1} IBA	**73c**	**4.60ab**	**55.20a**	**576.20ab**	**6.51a**	**93.49b**
	5 µM TDZ + 12 µl l^{-1} IBA	**84c**	**3.00b**	**23.00c**	**298.20b**	**6.01ab**	**93.99ab**
	Critical t value	**2.07**	**2.07**	**2.07**	**2.07**	**2.07**	**2.07**
'Ganymed'							
	DI Water	**100a**	3.80a	**27.13b**	480.30a	4.55a	95.45a
	5 µM TDZ + 4 µl l^{-1} IBA	**79b**	4.60a	**45.86a**	592.30a	4.95a	95.05a
	5 µM TDZ + 8 µl l^{-1} IBA	**33c**	3.70a	**37.67ab**	390.00a	4.39a	95.60a
	5 µM TDZ + 12 µl l^{-1} IBA	**79b**	3.70a	**34.00ab**	460.70a	4.94a	95.06a
	Critical t value	**2.08**	ns	**2.08**	ns	ns	ns
'Katinka'							
	DI Water	**100b**	**3.10b**	54.71a	**457.00b**	4.98a	95.01a
	5 µM TDZ + 4 µl l^{-1} IBA	**117a**	**4.80a**	66.38a	**860.40a**	4.78a	95.22a
	5 µM TDZ + 8 µl l^{-1} IBA	**92bc**	**4.00ab**	68.00a	**554.60ab**	5.39a	94.61a
	5 µM TDZ + 12 µl l^{-1} IBA	**78c**	**4.20ab**	68.67a	**704.30ab**	4.95a	95.04a
	Critical t value	**2.06**	**2.06**	2.06	**2.06**	ns	ns

TDZ treatment without IBA inhibited root formation (data not shown). Means separated by Student's *t* test ($P = 0.05$). For each cultivar, means followed by the same letter(s) within columns are not significantly different. N=8.

2.4 Discussion

2.4.1 Methods

New five *Pelargonium* cultivars susceptible to leaf yellowing were used. The goal was to find if various genotypes respond in the same way with respect to leaf colour, leaf chlorophyll content and rooting potential after exposing them to various treatments. Generally, all cultivars showed similar trends after treatments with respect to leaf chroma, leaf hue and chlorophyll content but variations were observed in root induction. Only three out of five cultivars rooted after treating them with TDZ followed by application of IBA in the rooting solutions. This could be attributed to variable environmental conditions in greenhouses (light, temperature and RH) since experiments were conducted at different times. Moreover, variable environmental conditions are known to affect the balance of endogenous levels of phytohormones in mother plants. Consequently, this could have influenced the biosynthesis of leaf chlorophyll, colour pigments and rooting process of cuttings either positively or adversely (Kelen and Ozkan, 2003).

In addition, Kadner and Druege (2004) attributed variation in rooting of 'Mitzou' zonal *Pelargonium* cuttings to environmental factors. Kadner and Druege (2004) argued that spring and early summer temperatures could have raised the leaf temperatures causing a higher degree of tissue dehydration during rooting period, which is known to reduce root regeneration capability of cuttings (Loach, 1988). Use of growth chambers and carrying experiments in one season would greatly reduce data variation. Furthermore, cuttings of 40 clones of Norway spruce tested on seven contrasting sites in Lower Saxony, Germany showed a high clone-site interaction. This could be reduced by selection of stable clones over a wide range of environments (Clair and Kleinschmit, 1986).

Chlorophyll determination according to Lichtenthaler (1987) elucidated clear cut effects among levels of various treatments as compared to Chroma meter (that determines leaf chroma and hue). Thus, Chroma meter could not detect differences between control and 0.5 µl l^{-1} ethylene or 5 and 10 µM TDZ throughout the experimental period. The same pattern was observed between 25 and 50 µM ABA except leaf chroma in 'Ganymed' after four days and 'Katinka' for the whole period of study (Fig. 3C, D). Additionally, both methods had similar results after storing the cuttings in the dark for 4 days. Moreover, Chroma meter could detect changes in leaf chroma after treatment with TDZ for two days that was not apparent in most

cultivars from chlorophyll extraction method. Leaf chroma and hue have been shown to be correlated to leaf chlorophyll and indicates a general trend of what is happening to the chlorophyll content (Steet and Tong, 1996). Overall, extraction of chlorophyll though very accurate is tedious and time consuming as opposed to use of Chroma meter, which is fast, efficient and user friendly but it is expensive. Furthermore, hydroponics rooting system was used for it is easy to evaluate roots compared to other media that are laborious, costs more time and leads to lose of roots during washing.

2.4.2 Results

Application of exogenous ethylene, ABA or dark storage accelerated senescence of the leaves of *Pelargonium* cuttings as expressed by visible onset of leaf yellowing (Figs. 2A, B, C). Additionally, these treatments increased leaf chroma and decreased leaf hue which is used to quantify a decrease in green colour of the leaves as they turn yellow (Steet and Tong, 1996). Furthermore, ethylene, ABA and dark storage hastened chlorophyll degradation (Figs. 6A, B, C, D, E). It is therefore inferred that chlorophyll degradation occurred leading to a decrease in leaf chlorophyll content with time. Chlorophyll breakdown in senescing leaves occurs via to a controlled, energy dependent process, which allows apoproteins complexed with chlorophyll to be broken down in order to salvage nitrogen (Matile *et al.,* 1996). Moreover, the results (leaf chroma and hue values) of the present study agree with those of Roberts *et al.* (1985), Purer and Mayak (1989) in that ethylene has been shown to induce premature leaf yellowing in many plants as a result of accelerated chlorophyll degradation.

Application of ethylene action inhibitor (1-MCP) retarded storage-induced leaf yellowing in zonal *Pelargonium* (Serek *et al.,* 1998; Kadner and Druege, 2004), implying ethylene action is involved in storage-induced leaf senescence. Additionally, storing cuttings shortly after harvest leads to water stress (Schatz, 1982) and accumulation of wound-ethylene in the packing material (Kadner *et al*., 2000), which promotes senescence as evidenced by leaf chlorosis (Schatz, 1982; Roberts *et al.* 1985). Moreover, during storage cuttings are exposed to darkness and low humidity that causes chlorosis (Behrens, 1988; Wang, 1987). Darkness stimulates senescence of green tissues (Thimann, 1980). In contrast, Zacarias and Reid (1990) found dark-induced leaf yellowing did not require the action of ethylene. Moreover, the levels of ABA increases in water stressed plants leading to leaf chlorosis (Aharoni *et al.*, 1977)

showing interaction of hormones in regulation of leaf senescence. Thus, it is evident that during storage, other ethylene-independent processes are also involved in leaf yellowing.

TDZ treatment delayed onset of leaf senescence, increased leaf hue and decreased leaf chroma (Figs. 4A, B, C, D). This effect on hue and chroma is because treated leaves remained green while those of untreated controls turned yellow (Fig. 2D). Additionally, TDZ retarded leaf chlorophyll degradation by maintaining or slightly increasing it as compared to controls (Figs. 4E, F). Cytokinins reduce leaf yellowing in ornamental plants (Richmond and Lang, 1957; Mutui *et al*., 2001) because they are involved in chlorophyll biosynthesis (Zavaleta-Mancera *et al.,* 1999). Chory *et al.* (1994) showed that if etiolated leaves of *Arabidopsis* are treated with a cytokinin before being illuminated, they form chloroplasts with more extensive grana. Upon illumination, chlorophyll and photosynthetic enzymes were synthesized at a faster rate, implying cytokinins regulate synthesis of photosynthetic pigments and proteins.

Zavaleta-Mancera *et al.* (1999) reported that cytokinins activate NADH protochlophyllide reductase, an enzyme involved in chlorophyll biosynthesis, and reduce chlorophyll degradation in tobacco leaves. Exogenous cytokinins promoted regreening in yellow leaves (Zavaleta-Mancera *et al.,* 1999). Transgenic plants of *Nicotiana tabacum* and *Arabidopsis thaliana* with a gene inserted that increases cytokinin synthesis did not exhibit leaf yellowing (Zavaleta-Mancera *et al*., 1999). Moreover, Ferrante *et al*. (2002a, 2002b) reported that TDZ prevented leaf yellowing in *Alstroemeria* cut flowers, cut Tulips and cut Chrysanthemum ‘Regan bianco’ by inhibiting chlorophyll degradation.

Ethylene enhanced root induction, but reduced the capacity of cuttings to continue growth of induced roots as expressed by decreased number of roots per cutting, root length, root fresh weights and percent water content (Table 1). The fact that ethylene-insensitive transgenic petunia formed few adventitious roots (Clark *et al*. 1999) and 1-MCP (ethylene action inhibitor) reduced rooting in *Pelargonium* cuttings (Serek *et al*., 1998) support the view that ethylene is involved in adventitious rooting. Kadner and Druege (2004) observed promotion of root formation in stored zonal *Pelargonium* ‘Mitzou’ cuttings after application of ethylene. The reduced capacity of induced roots to grow could be attributed to stress response mechanisms induced by high levels of ethylene. Mensuali-Sodi et al. (1995) showed the rooting of tomato cotyledon and lavandin micro-cuttings was inhibited when endogenous ethylene levels were either increased or decreased. This implies that ethylene was promoting

rooting over a narrow range of concentrations. These concentration-dependent effects of ethylene (Ma *et al*., 1998) and difference in sensitivity of experimental plant organs to ethylene at different stages of development (Clark *et al.,* 1999) may account for the conflicting results in literature.

In contrast, 0.5 µl l^{-1} ethylene increased the percent root dry matter accumulation in 'Greco' and 'Surfing' (Table 1). Similarly, ethylene-insensitive Never ripe (*Nr*) tomato plants produced more below-ground root mass but fewer above-ground adventitious roots than Pearson wild-type plants (Clark *et al*., 1999). Clark *et al*. (1999) suggested that ethylene does not play a major role in root formation of plants established in soil and growing under optimal growth conditions. It is therefore inferred that low ethylene level used in this study could have played a similar role. Moreover, although there are reports that ethylene may be involved in adventitious rooting, the literature is conflicting. Ethylene and ethylene-releasing compounds have been reported to inhibit adventitious root formation in pea cuttings (Nordström and Eliasson, 1984) and faba bean (Khalafalla and Hattori, 2000), promote in mung bean cuttings (Robbins *et al*., 1985) or have no effect in mung bean cuttings (Mudge and Swanson, 1978).

Exogenous application of 50 µM and 100 µM ABA reduced the ability of *Pelargonium* cuttings to regenerate roots. Furthermore, ABA decreased the number and length of the roots, root fresh weight and water content. Applied ABA can inhibit ethylene production from various organs in a range of species (Tan and Thimann, 1989; Spollen *et al*., 2000). Conversely, Wright (1980) showed that pre-treatment with ABA prevents increase in ethylene production caused by wilting of excised wheat leaves. In the current context exogenous ABA may restrict ethylene production (Spollen *et al.,* 2002) play a role in *Pelargonium* root formation. In contrast, 25 and 50 µM ABA unexpectedly increased rooting proportion in 'Greco' (Table 2). ABA has been shown to stimulate root growth in *Phaseolus coccinensis* (Hartung and Abou-Mandour, 1980) and results obtained in the current study could be ascribed to a greater partitioning of assimilates to the growing roots in the nutrient solution at the expense of vegetative growth (Blum and Sullivan, 1997).

Consistent with results of this study, Blum and Sullivan (1997) found a 30% increase in root biomass of dwarf spring wheat (Rht3) grown under atmospheric desiccation stress as compared to the control. ABA, a stress hormone maintained root growth and reduced shoot growth thus increasing root/shoot ratio (Sharp, 1990). Furthermore, Spollen *et al*. (2002)

demonstrated that ABA accumulation plays an important role in the maintenance of root elongation in maize. This role was profound at low water potential, when ABA restricted ethylene production. Therefore, Spollen *et al*. (2002) concluded that restriction of ethylene production may be a widespread function of ABA. Moreover, in *flacca* mutant of tomato, it was shown that ethylene production could be restored to normal levels with exogenous ABA (Tal *et al*., 1979). However, it was uncertain whether the increase in ethylene evolution was a direct result of ABA deficiency or it was indirect effect of decreased plant water status.

Storing cuttings for four days in the darkness had no effect on rooting percentage but it decreased all the other investigated root parameters (Table 3). Furthermore, storing *Pelargonium* 'Mitzou' at 20^{o}C decreased the number of roots per cutting (Kadner and Druege, 2004). The results of the present investigation suggest that the amount of ethylene released by zonal *Pelargonium* cuttings enclosed in un-perforated polyethylene bags (Kadner *et al*., 2000) exceeds the threshold level required for optimal root production. Based on studies with ethylene and ethylene inhibitors, Jusaitis (1986) suggested that low (10-fold basal ethylene) concentrations of ethylene are required for rooting of mung bean cuttings, whereas too high (1000-fold basal ethylene) concentrations have inhibitory effect.

In accordance with present findings, short-term storage inhibited rooting of *Pelargonium* cuttings (Serek *et al*., 1998). In addition, ethylene was found to inhibit adventitious root formation in pea cuttings (Nordström and Eliasson, 1984). Contrary, it was found to stimulate root formation in mung bean cuttings (Robbins *et al*., 1985). With respect to stress induced by water deficiency to cuttings during storage, Kage *et al*. (2004) reported that specific root length of cauliflower was lower under drought stress conditions leading to a higher dry matter deposition in the fine root fraction. Moreover, Eisenberg *et al*. (1978) reported a decrease in quality and rooting abilities for many ornamental cuttings after storage.

It was observed that low (4 µl l^{-1}) IBA level induced maximal (100%) root induction in 'Ganymed' (Table 4). IAA enhances the synthesis of enzymes that induce hydrolysis of starch and other nutrients, so IAA seem to be involved directly with initiation of roots and formation of vascular tissue (Kracke *et al*., 1981). IAA also induces ethylene synthesis in many plant species and tissues (Kawase, 1971). Therefore, the current results suggest that ethylene induced by auxins may account for the observed root promoting activity of IBA. Higher (8 and 12 µl l^{-1}) IBA concentrations decreased the proportion of rooted cuttings and other

investigated root parameters. It is possible that this observation was due to higher demand for carbon allocation as a result of increased adventitious root formation in *Pelargonium* cuttings (Clark *et al*., 1999). Furthermore, Mudge (1988) hypothesised that if a low saturating concentration for ethylene-stimulated rooting exists, then auxin-stimulated ethylene production above this level would have no additional effect on rooting.

Since auxins are known to induce ethylene biosynthesis (Kawase, 1971), numerous attempts (Geneve and Heuser, 1982, Mudge, 1988) have been made to determine if interactions exists between auxin and ethylene during adventitious root formation and development. However, there has been no clear correlation between the two (Geneve and Heuser, 1982, Mudge, 1988). Reports of the variable rooting response of many plant systems to ethylene compared with ubiquitous reports of auxin-stimulated rooting have suggested that ethylene is less often a limiting factor or is less directly involved in the rooting process than auxin (Mudge, 1988). Overall, the promotive effect of auxin on adventitious rooting is influenced by ethylene responsiveness (Clark *et al*., 1999).

TDZ treatment severely inhibited root induction (Fig. 7D), but this was effectively offset in 'Fire', 'Ganymed' and 'Katinka' by application of IBA (Fig. 7F, Table 5) in the rooting solutions. This result may be explained in that TDZ is very stable in *Pelargonium* leaves leading to root initiation inhibition. Mok and Mok (1985) found that [^{14}C]-TDZ was not substantially broken down in *Phaseolus lunatus* callus tissue over 33 days. Mok and Mok (1985) concluded that TDZ itself and not its catabolites was stimulating the physiological responses. Ferrante *et al.* (2003) reported that TDZ inhibited rooting in cut Chrysanthemum 'Regan giallo'. Additionally, synthetic cytokinins inhibited root initiation in Rhododendron stem cuttings (Pierik and Steegmans, 1975). In *Arabidopsis*, a down-regulated cytokinin receptor mutant and a loss-of-function allele of a cytokinin signalling element both have longer roots than the wild type (Inoue *et al.* 2001; Sakai *et al*., 2001). Additionally, transgenic tobacco engineered to over express cytokinin oxidase (and thus to have lower levels of cytokinin) also has longer roots than its wild type counterpart (Werner *et al*., 2001). These findings suggest that high levels of endogenous cytokinins regulate root elongation negatively. Furthermore, Murch and Saxena (2001) found that the TDZ molecule remained intact in both a free and conjugated form within the hypocotyls tissues of *Pelargonium x hortorum* and this suggests that TDZ exposure enhances accumulation and translocation of auxin within tissues.

The fact that TDZ treated *Pelargonium* cuttings followed by application of IBA at 4 µl l^{-1} restored their rooting abilities as evidenced by 100% root induction in 'Ganymed' is a clear evidence of a central role of auxins in adventitious root formation. Similarly, 4 µl l^{-1} IBA produced comparable number of roots, root lengths and fresh weights in 'Fire', 'Ganymed' and 'Katinka'. Also, TDZ increased ethylene production in *Pelargonium* cuttings (Fig. 12), geranium hypocotyl cultures (Hutchinson *et al*., 1997), mung bean hypocotyl segments (Suttle, 1984) and suspension cultures (Yip and Yang, 1986). This led Hutchinson *et al*. (1997) to hypothesise that TDZ enhanced ethylene production leading to elevated levels, which was inhibitory to somatic embryogenesis. Other reports indicated that when NAA was applied alone after decapitation of pea cuttings, it stimulated rooting, probably by being an auxin and partly by inhibiting accumulation of cytokinins at the base (Koukourikou-Petridou and Bangerth, 1997). Koukourikou-Petridou and Bangerth, (1997) concluded that adventitious root formation is a complex intrinsic balance between auxins and cytokinins. Generally, a high auxin/low cytokinin ratio favours adventitious root formation and a low auxin/high cytokinin ratio favours adventitious bud formation (Bouza *et al*., 1994). In the current context, it can be concluded that the yet unidentified role of TDZ in ethylene biosynthesis, possibly as inhibitor of ethylene perception through over-expression of ethylene receptor genes (Figs. 17, 18), may at least in part, account for the observed difficulty in rooting of TDZ-treated cuttings.

2.5 Conclusion

Ethylene, ABA and dark storage accelerated leaf senescence and reduced rooting percentage of cuttings whereas TDZ delayed leaf senescence and inhibited root formation. Promotion of leaf senescence by ABA was indirectly through increased ethylene formation. Dark storage evidently had similar effects probably because it is a form of stress that led to enhanced ethylene evolution. TDZ, despite having root inhibitory effects that need to be offset by application of IBA in rooting solutions, is able to counteract deleterious effects of dark storage, ethylene and ABA. Thus TDZ treatment had a marked beneficial effect of delaying the onset of leaf yellowing in *Pelargonium* cuttings during storage and shipment. For practical purposes of TDZ, genotypes should be tested to determine optimum levels of using TDZ and IBA for preventing leaf senescence and inducing rooting, respectively. In future work, testing efficacy of novel aromatic cytokinin that are easily metabolised in plant tissues and have potential to prevent leaf yellowing without inhibiting root formation will be done.

3.0 Isolation, Characterization and Expression studies on *ACC* synthase and Ethylene Receptor (*ETR1*) genes in *Pelargonium*

Abstract

The effects of TDZ, ethylene, ABA or dark storage on *ACC* synthase and ethylene receptor (*ETR1*) genes during *Pelargonium* leaf senescence were studied. Genomic DNA and total RNA were isolated from roots, stems, leaves, flower buds, petals and pistils of 'Katinka' and / or 'Ganymed' for PCR and One step RT-PCR, respectively. Degenerate primer pair was used to amplify three DNA fragments using genomic DNA from 'Katinka' leaves. Sequence analysis of two novel partial putative ACC synthases led to their characterisation and designation as *PzACS3* and *PzACS4*. *PzACS3* is 590 bp long with 374 bp coding region and two introns, 83 and 133 bp long, respectively. Additionally, *PzACS4* is 745 bp long with 374 bp exon and two introns, 169 and 202 bp long too. Gene-specific primers for the *ACC* synthase and ethylene receptor (*ETR1*) genes were constructed and synthesized. Expression studies were done using a One step RT-PCR. Conversely, *PzACS1* and *PzACS2* were expressed in most of tissues examined in 'Katinka' except in roots and petals, respectively. In contrast, *PzACS2* was not detected in 'Ganymed' leaves. *PzACS3* and *PzACS4* transcripts were respectively expressed or undetectable in roots while *PzETR1* was strongly expressed in roots and flower buds whereas *PzETR2* was constitutively expressed in all tissues. Tissue specific gene expression patterns suggest they have different roles in ethylene biosynthesis and signaling. Also, transcripts of these genes were induced in a treatment-specific fashion and correlated positively with ethylene production by cuttings after 4 days in various treatments except for 2 µl l^{-1} ethylene which had inhibitory effect. Ethylene slightly down regulated the expression of *PzACS1* transcripts. Additionally, 100 µM ABA and dark storage increased *PzACS1* and *PzACS2* mRNAs, respectively. This was due to either stress-induced ethylene that occurs after dark storage or ABA treatment. Moreover, for the first time, TDZ was shown to strongly induce expression of *PzETR1*, possibly by increasing the amount of ethylene receptors via up-regulation of *PzETR1* transcripts, thus reducing sensitivity of leaves to ethylene with the concomitant beneficial effect of delaying the onset of leaf yellowing.

Key words: 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, Dark stress response, Ethylene receptor (*ETR1*) genes, Gene expression, *Pelargonium zonale*, Phytohormones, TDZ

3.1 Introduction

3.1.1 Overview

Phytohormones are essential for integrating many aspects of plant development and responses to the environment. Ethylene, a simple readily diffusible gaseous hormone is involved in a variety of plant growth, development and stress related processes including tissue senescence, seed germination, leaf abscission, stem or root elongation, root hair development, epinasty, fruit ripening and flower fading (Abeles *et al*., 1992). *Pelargonium zonale* hybrids are classified as ethylene sensitive (Woltering, 1987) and ethylene leads to abscission of leaves and flowers. Conversely, exogenous application of ethylene enhances the senescence process while inhibition of ethylene synthesis or action slows senescence (Reid and Wu, 1992). The production of ethylene is tightly regulated by internal signals during development and in response to environmental stimuli from biotic (e.g. pathogen and fungal infection) and abiotic stresses such as wounding, anaerobiosis, ozone, drought, chilling, heavy metals, auxin, ripening and senescence processes (Wang *et al.*, 2002). Ethylene is found in the atmosphere as a pollutant and is also internally biosynthesized in plants. Different plant species respond differently to varying levels of ethylene (Abeles *et al*., 1992). To understand the roles of ethylene functions, it is important to know how this gaseous hormone is synthesized, how its production is regulated and how the signal is transduced (Wang *et al.*, 2002).

Application of ethylene or its metabolic precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) is known to induce a "triple response" trait in dark-grown (etiolated) dicotyledonous seedlings (Bleecker *et al*., 1988). This is characterised by exaggerated curvature of the apical hook, radial swelling of the hypocotyls and shortening of the hypocotyls and roots. Over the past decade, the triple response phenotype has been used to screen mutants that are defective in ethylene responses (Bleecker *et al*., 1988). Etiolated *Arabidopsis* seedlings with minor or no phenotypic response upon ethylene application are termed ethylene-insensitive (*ein*) or ethylene resistant (*etr*) mutants. Other mutants that display constitutive triple response in the absence of ethylene have also been identified (Kieber *et al*., 1993). They are grouped based on whether or not the constitutive triple response can be suppressed by inhibitors of ethylene perception like (1-MCP or STS) and / or biosynthesis (AVG). Those mutants unaffected by these inhibitors are termed constitutive triple-response (ctr) whereas mutants whose phenotype reverts to normal physiology are termed ethylene-overproducer (*eto*) mutants, which are defective in the regulation of ethylene biosynthesis (Wang *et al.*, 2002).

3.1.2 Ethylene biosynthesis

The genetic hierarchy among ethylene biosynthesis and signalling pathway components in *Arabidopsis* has been established by epistasis analysis using etr, ctr or eto mutants (Solano and Ecker, 1998). Ethylene is biosynthesized from methionine, which in addition to being an essential building block of protein synthesis, is converted into S-adenosyl-methionine (S-AdoMet) by the enzyme AdoMet synthase (Yang and Hoffman, 1984). The first committed and rate-limiting step of ethylene synthesis is the conversion of S-AdoMet to ACC (the immediate precursor of ethylene) by the enzyme ACC synthase (Yang and Hoffman, 1984). Lastly, ACC is converted into ethylene by ACC oxidase. The first successes in molecular cloning of the ACC synthase genes in zucchini were reported by Sato and Theologis (1989) and for ACC oxidase genes in tomato by Hamilton *et al*. (1991). The observations that expression of the ACC synthase genes are highly regulated by a variety of signals and that active ACC synthase is labile and present at low levels suggest that ethylene biosynthesis is tightly controlled (Wang *et al.*, 2002).

The ethylene biosynthetic enzymes, AdoMet synthase, ACC synthase and ACC oxidase are encoded by multi-gene families (Bleecker and Kende, 2000; Rottmann *et al*., 1991). For instance, ACC synthase families in *Lycopersicon esculentum* consist of at least nine members (Rottmann *et al*., 1991) whereas in *Arabidopsis thaliana* comprise of twelve putative genes (Liang *et al*., 1992; The Arabidopsis Genome Initiative, 2000). Because ACC synthase, a pyridoxal phosphate containing enzyme (Bleecker and Kende, 2000), plays a central role in ethylene biosynthesis, its regulation has been intensively investigated (Wang *et al.*, 2002). Also, its activity is regulated at the transcriptional (Rottmann *et al*., 1991) and post-transcriptional levels (Vogel *et al*., 1998) and it is believed that enhanced transcription is the main regulatory step of these enzymes' activities, though post-transcriptional regulation is operational too (Vogel *et al*., 1998). An emerging paradigm is that different isoforms of ACC synthase are differentially regulated (Peck and Kende, 1998a).

3.1.3 Ethylene signal transduction

Molecular mechanisms underlying ethylene signalling, including the identification of the genes encoding the ethylene receptor such as *ETR1*-like genes and several downstream signalling elements have been isolated and characterized in *Arabidopsis* (Chang *et al*., 1993;

Chao *et al*., 1997; Solano *et al*., 1998). Consequently, the translation product of *ETR1* has three membrane spanning regions at N-terminal hydrophobic domains where ethylene binding occurs (Schallar *et al.,* 1995), and a well conserved histidine kinase domain at the C-terminal part of the protein (Chang *et al*., 1993). Five *ETR1*-like genes; *ETR1* (Chang *et al*., 1993), *ERS1* (Hua *et al.,* 1995), *ETR2* (Sakai *et al*., 1998), *EIN4* and *ERS2* (Hua *et al*., 1998) have been identified in *Arabidopsis*. Hua and Meyerowitz (1998) classified *ETR1* and *ERS1* into *ETR1*-like subfamily, which have three transmembrane domains in the N-terminus. Additionally, *ETR2*-like subfamily comprise of *ETR2, ERS2* and *EIN4*, which have four hydrophobic transmembrane domains in the N-terminus. Among these receptors, only *ETR1, ETR2* and *EIN4* contain a receiver domain that shows similarity to the bacterial two component system (Chang *et al*., 1993; Hua *et al.,* 1995). The expression of *ETR1* and *ERS1* from *Arabidopsis* in yeast provides a high affinity binding site for ethylene, hence *ETR1* acts as an ethylene receptor (Schallar *et al.,* 1995).

Point mutations in transmemebrane domain of *ERS1* (Hua *et al.,* 1995) and *NR* (Lanahan *et al*., 1994) cause insensitivity to ethylene in *Arabidopsis* and tomato, respectively, indicating these homologues share a common function with *ETR1*. Moreover, four mutants *etr1-1, etr1-2, etr1-3* and *etr1-4* were modified by a single amino acid of three N-terminal transmembrane domains in the *ETR1* protein isolated in *Arabidopsis* and all were found to be insensitive to ethylene. Introduction of one of these genes into wild type *Arabidopsis* also caused a loss of sensitivity to ethylene (Chang *et al*., 1993). Furthermore, loss-of-function ctr1 mutants exhibit constitutive ethylene responses, which suggest that *CTR1* acts as a negative regulator of ethylene responses. The predicted *CTR1* protein is a serine-threonine protein kinase that is most closely related to the Raf protein kinase family (Kyriakis *et al*., 1992). Double mutant analysis indicates that *CTR1* acts downstream of *ETR1* (Kieber *et al*., 1993).

The current paradigm is that ethylene receptors, which are located in the cytoplasmic-cell membrane, are responsible for ethylene perception (Wang *et al*., 2002). According to a model by Klee (2002), Ciardi and Klee (2001), *Arabidopsis*' *ETR1* functions as a membrane-associated homodimer linked by two disulfide (S-S) bonds (Schallar *et al.,* 1995) as shown in (Fig. 8). Wild-type receptor without ethylene binding would cross phosphorylate a conserved histidine within the kinase region. This cross phosphorylation would initiate a signaling cascade, which suppresses the ethylene response. Ethylene binding occurs at the N-terminal transmembrane domain and *ETR1* exhibits a copper co-factor mediated high affinity ethylene

binding (Schallar *et al.,* 1995). *Arabidopsis* RESPONSIVE-TO-ANTAGONIST (*RAN1*) gene is involved in the delivery of copper to the ethylene receptor (Hirayama *et al*., 1999). Interestingly, silver also binds to *ETR1* and mediates ethylene binding. Additionally, silver is widely used as inhibitor of ethylene perception probably by displacing copper in the active site of the receptor complex (Ciardi and Klee, 2001). Moreover, 1-MCP binding to the receptor helps to block ethylene perception (Sisler and Serek, 1997).

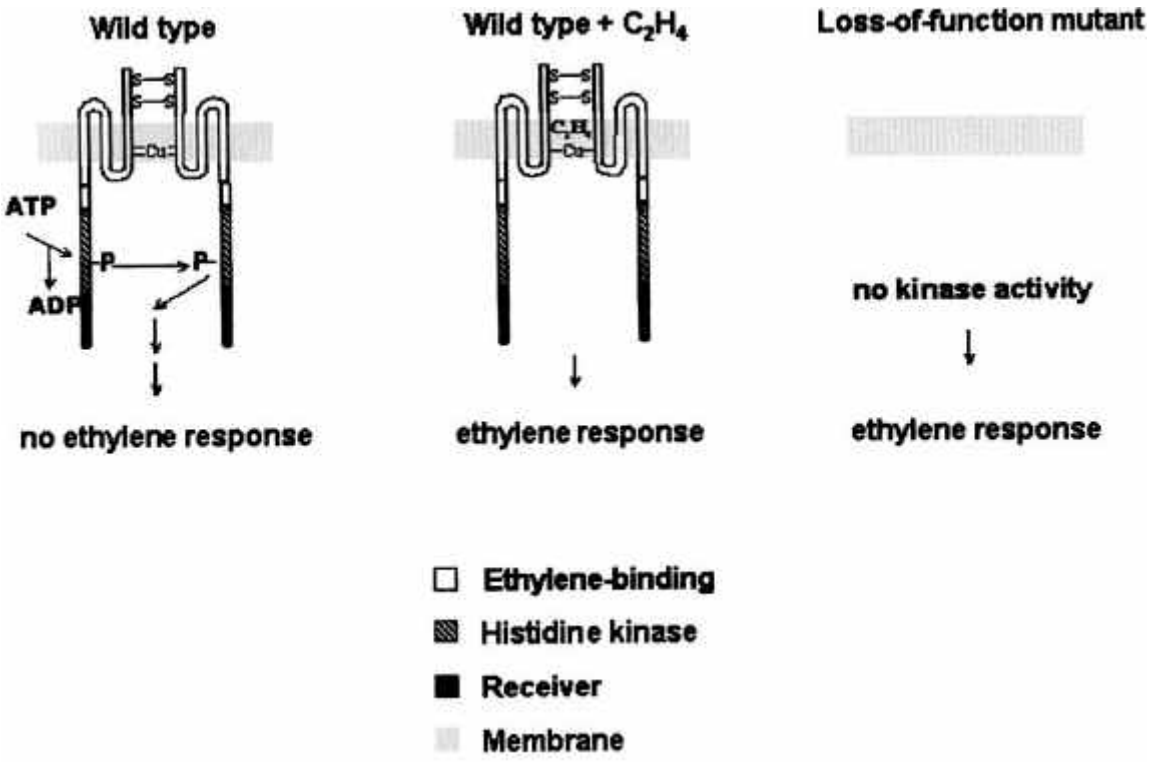

Fig. 8. Model for role of ethylene receptors in ethylene signalling (Ciardi and Klee, 2001; Klee, 2002).

Binding of ethylene causes a conformational change in the receptor, which suppresses kinase activity (turns the receptor off) thus relieving the downstream block on signal transduction and allowing an ethylene response to occur. Loss-of-function mutations in multiple ethylene receptors would eliminate kinase activity and removes active suppression of ethylene response. Additionally, partial loss-of-function mutants should require less ethylene than wild-type to achieve an ethylene response since there is less receptor on a molar basis to inactivate (Ciardi and Klee, 2001; Klee, 2002).

According to a model by Wang *et al.* (2002), in the absence of an ethylene signal, ethylene receptors activate a Raf-like kinase, *CTR1* (Kyriakis *et al.*, 1992) which in turn negatively regulates the downstream ethylene response pathway, possibly through a mitogen-activated

protein (MAP) kinase cascade (Fig. 9). Furthermore, genetic epistasis analysis of ethylene response mutants have shown that ETHYLENE INSENSITIVE (*EIN2)* acts downstream of *CTR1* and upstream of *EIN3* (Alonso *et al*., 1999). Also, null mutations in *EIN2* result in the complete loss of ethylene responsiveness throughout plant development, suggesting it is an essential positive regulator in ethylene signalling pathway (Alonso *et al*., 1999). Therefore, binding of ethylene inactivates the receptors, resulting in deactivation of *CTR1* (Kieber *et al*., 1993), which allows *EIN2* to function as a positive regulator of the ethylene pathway (Alonso *et al*., 1999).

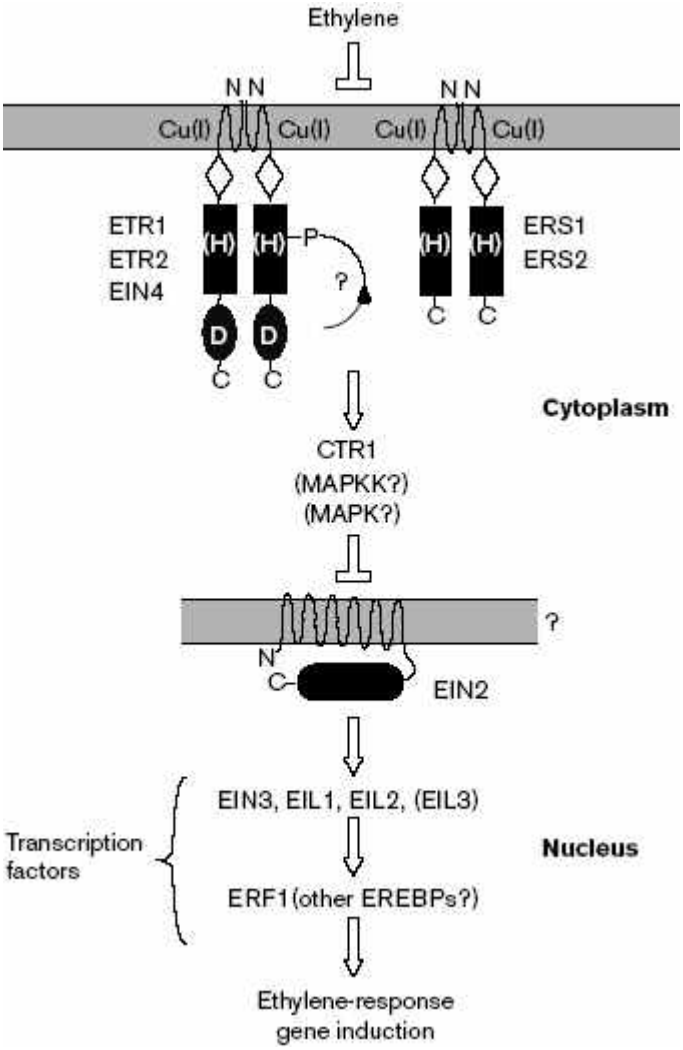

Fig. 9. Ethylene signal transduction pathway model in *Arabidopsis* (Chang and Shockey, 1999).

EIN2 contains the N-terminal hydrophobic domain similar to the Nramp metal transporter proteins and the novel hydrophilic C terminus (Alonso *et al*., 1999). Consequently, it positively signals downstream to the *EIN3* (Chao *et al*., 1997) family of transcription factors located in the nucleus. A search for target promoters for the *EIN3* family of proteins led to the identification of the primary ethylene response element binding proteins (*EREBPs*) (Solano *et*

al., 1998). *EIN3* binds to the promoter of *ERF1* gene and activates its transcription in an ethylene-dependent manner. Transcription factors, *ERF1* and other *EREBPs* can interact with the GCC box in the promoter of target genes and activate downstream ethylene responses (Yamamoto *et al*., 1999).

3.1.4 Expression of *ACC* synthase and ethylene receptor (*ETR1*) genes

Expression of selected members of the multi-gene ACC synthase family in higher plants is induced by a diverse group of external and internal ethylene inducers. For instance in *Arabidopsis*, *AtACS2* is induced by cycloheximide, wounding and 2 h of ethylene exposure (Liang *et al*., 1996). However, ethylene-induced expression gradually decreases with prolonged ethylene exposure, suggesting negative feedback regulation of *AtACS2* (Liang *et al*., 1996). *AtACS*3 does not show ACC synthase activity in either bacterial or yeast expression systems (Liang *et al*., 1996) and is believed to be a pseudogene resulting from a partial duplication of *AtACS1* (Wang *et al.*, 2002). *AtACS4* is induced in seedlings by cycloheximide, idoleacetic acid and wounding (Liang *et al*., 1992). Also, *AtACS5* is induced by lithium chloride (Liang *et al*., 1996). Additionally, *AtACS*6 can specifically be induced by cyanide treatment, exposure to ozone in light-grown leaves as well as by cycloheximide, idoleacetic acid and ethylene (Liang *et al*., 1992; Smith and Arteca, 2000). Moreover, *AtACS10* promotes flowering of *Arabidopsis* in response to light (Samach *et al*., 2000). Because cycloheximide treatment induces most of the ACC synthase isoforms, the implication is that ACC synthase transcripts are short-lived and negatively regulated by some unknown labile repressor(s) (Liang *et al*., 1992). Alternatively, cycloheximide treatment could possibly result in retention of mRNA on the ribosome thus relatively increasing the steady state of ACC synthase mRNA (Wang *et al.*, 2002).

Most of ethylene receptor (ETR) genes isolated from *Arabidopsis* (Tieman *et al*., 2000) are homologous to those of geranium (Dervinis *et al*., 2000), carnation (Shibuya *et al*., 2002; Nagata *et al*., 2000), rose (Müller *et al*., 2000a; Müller *et al*., 2000b) and *Delphinium* (Kuroda *et a*l., 2003). Ethylene receptors have been shown to act as negative regulators of ethylene responses in *Arabidopsis* (Hua and Meyerowitz, 1998) and tomato (Tieman *et al*., 2000). Additionally, these genes are up regulated by both developmental and exogenous stimuli. For instance in geranium, *PhETR1* and *PhETR2* are expressed at moderate levels in leaves, pedicels, sepals, pistils and petals and at very low levels in roots (Dervinis *et al*., 2000). Their

expression at flower bud stage indicates that the amount of receptors is not indicative of the level of sensitivity of geranium florets to ethylene. Hence, Dervinis *et al*. (2000) concluded that perhaps the control of ethylene induced petal abscission in geranium florets may be mediated by another yet uncharacterised member of the *PhETR* gene family, at the post transcriptional level or through a downstream component of signal transduction pathway.

Expression of *RhETR1* and *RhETR3* mRNAs were distinctly higher in 'Bronze', a miniature rose cultivar with shorter flower life, than in long lasting 'Vanilla' (Müller *et al*., 2000a; Müller *et al*., 2000b), suggesting flower life in roses is a function of inherent amount of receptors (Müller *et al*., 2000b). Moreover, the highest expression of *RhETR1* transcripts was at bud stage and young open flowers in 'Bronze' and 'Vanilla', respectively (Müller *et al*., 2000a), indicating rose flowers respond to ethylene at early developmental stages. Additionally, *RhETR3* transcripts increased in senescing flowers of 'Bronze' while both *RhETR2* and *RhETR3* mRNAs in 'Vanilla' appeared to be constitutively expressed albeit at very low levels (Müller *et al*., 2000b). Also, *DC-ERS2* and *DC-ETR1* transcripts were expressed at considerable amounts in the petals, ovaries and styles of carnation flowers, at full-opening stage (Shibuya *et al*., 2002). However, the level of *DC-ERS2* mRNAs in petals decreased as flower senesced while it slightly increased in ovaries and was unchanged in styles whereas *DC-ERS1* transcripts were not detectable at any time (Shibuya *et al*., 2002). Moreover, in tomato, expression of *LeETR4* and *LeETR5* mRNAs were highly regulated among plant tissues with high levels in reproductive (flower buds and mature flowers) tissues whereas *LeETR1* was constitutively expressed in all tissues (Tieman and Klee, 1999).

In *Delphinium*, expression of *DI-ERS1* was proportional to endogenous ethylene produced by the florets, which in turn could have been perceived by the elevated *DI-ERS1* levels to cause flower senescence (Kuroda *et al*., 2003). In contrast, levels of *DC-ERS2* and *DC-ETR1* transcripts in carnation petals decreased inversely with the increase in ethylene production in untreated flowers but this decrease was independent of ethylene production, for those treated with 1, 1-dimethyl-4-(phenylsulphononyl) semicarbazide (DPSS) which blocks ethylene production (Shibuya *et al*., 2002). This implies that *DC-ERS2* and *DC-ETR1* are ethylene receptor genes responsible for ethylene perception in carnation and their expression during flower senescence is regulated in a tissue specific manner and independent of ethylene (Shibuya *et al*., 2002). Furthermore, expression of *RhETR1* preceded ethylene production by rose flowers but abundance of *RhETR3* transcripts increased during flower senescence in

'Bronze' indicating that ethylene response system is composed of multiple receptor types with overlapping patterns of expression (Müller *et al*., 2000b). Also, expression of *RhETR1* and *RhETR3* in 'Vanilla' was low despite having moderate ethylene production.

Transcript levels of *PhETR1* and *PhETR2* in pistils and receptacles were unaffected either by self-pollination or exogenous ethylene in *Pelargonium* (Dervinis *et al*., 2000) and in carnations petals, indicating these genes are not subject to up-regulation by ethylene (Shibuya *et al*., 2002). In contrast, exposing miniature rose flowers to ethylene increased expression of *RhETR1* (Müller *et al*., 2000a) and *RhETR3* (Müller *et al*., 2000b). On the other hand, expression of *RhETR2* transcripts increased after ethylene treatment in 'Bronze' only (Müller *et al*., 2000b). These results are in contrast to the standard model of ethylene signal transduction (Bleecker, 1999), whereby a reduction in the level of receptors would normally lead to increased ethylene sensitivity and vice versa. Nevertheless, in *Arabidopsis* leaves, transcript levels of *ERS1, ETR2* and *ERS2* genes were up-regulated by ethylene while it had no effect on *ETR1* and *EIN4* (Hua *et al*., 1998). These authors suggested that differential regulation of the receptor genes' expression may provide a mechanism for achieving differential sensitivities even in the same response under different conditions (Hua *et al*., 1998). ABA application increased expression levels of *RhETR2* and *RhETR3* in miniature rose (Müller *et al*., 2000) and induced ABA-responsive (rab) gene (Skriver and Mundy, 1990). Additionally, in *Arabidopsis*, *AtACS5* was induced by a low concentration of cytokinin in etiolated seedlings (Vogel *et al.,* 1998). Moreover, van Gysel *et al*. (1993) demonstrated that photo-regulated (bcb) gene was induced by darkness in *Arabidopsis*.

Because *ACC* synthase plays a central role in ethylene biosynthesis and subsequent regulatory effect in the senescence process (especially for ethylene sensitive plants like *Pelargonium zonale* hybrids*)*, isolation of new members of the *ACC* synthase gene family was carried out using a PCR method, based on amplification of genomic DNA fragments with degenerate primer pairs. Additionally, in order to better understand the pattern and control of the expression of *PzACS3* and *PzACS4* genes alongside other ethylene biosynthetic pathway genes (obtained from the gene bank), characterization of their distribution in roots, stems, leaves, flower buds, petals and pistils with respect to temporal and spatial regulation was investigated using RT-PCR method. Moreover, molecular studies on the effects of ethylene, ABA, dark storage and TDZ on accumulation of mRNAs encoding *ACC* synthase and *ETR1* genes were performed.

3.2 Materials and Methods

3.2.1 Plant material

Pelargonium zonale hybrids cultivars 'Katinka' and 'Ganymed' were grown as described in Section 2.2. In order to perform expression studies of the ethylene biosynthetic genes, various plant tissues were collected. Young (non-woody) stem tissues were used. Roots were collected after 1 month growth of cuttings under hydroponics. The second leaf below the apical region, which was dark green, not folded and wrinkled was used and referred to as fully expanded leaf. Flower buds were closed florets containing a calyx and a pedicel approximately 2 mm long. The age of the florets were determined by morphology. Petals and pistils were obtained from fully open (non-senescent) flowers that had slightly open stigmas ready for pollination. These plant tissues were put in 15 ml tubes (Sarstedt, Germany) and immediately frozen in liquid nitrogen. They were then ground in liquid nitrogen and stored at -80^{o}C deep freezer until extraction of DNA or RNA.

3.2.2 Ethylene, ABA, TDZ and dark storage treatment

Terminal *Pelargonium* cuttings with three fully expanded leaves were harvested and their foliage were completely immersed in 20 µM TDZ or 100 µM ABA solutions for 1 minute as described in Section 2.2. For control, dark storage and ethylene, the foliage on cuttings were immersed in deionised water containing 0.2% (v/v) Tween 20 and laid on absorbent paper to dry for 30 minutes. Then, cuttings for ethylene treatment were placed in sealed 54 l glass chambers and 0.11 ml pure ethylene gas was injected with a hypodermic syringe to give 2 µl l^{-1} for 6 hours and thereafter ventilated for 1 hour before being placed in glass bottles fitted with rubber septum caps.

3.2.3 Ethylene production determination

The fresh weights of all the cuttings were determined. Two cuttings were placed in each of 250 ml glass bottles and sealed with rubber septum caps. For dark storage, the glass bottles were placed randomly into boxes and stored in the dark. Gas samples (0.5 ml) were withdrawn with a syringe from the headspace after every 12 hours for 4 days. Ethylene levels in the samples were measured by gas chromatograph and expressed as nanolitres per litre per gram of fresh weight as described in Section 2.2.

3.2.4 Database analyses and primer design

Sequence comparison between known ACC synthase sequences of *Cucumis sativus* L from the NCBI gene bank (http://www.ncbi.nlm.nih.gov) was done according to Mibus (2003). Degenerate primers that allow for amplification of unknown ACC synthase genes were developed using homologous regions of *CsACS1* (AB032937), *CsACS2* (AB032938) and *CsACS3* (AB006805) sequences. The nucleotide and amino acid sequences; 5'TT(CT)CA(AG)GA(CT)TA(CT)CA(CT)GGI(CT)TICC'3 and FQDYHGLP were used for the sense primer ACSd2s whereas 5'GTICCIA(AG)IGG(AG) TTIGAIGG(AG)TT'3 and NPSNPLGT were used for anti sense primer ACSdas, respectively. Where; () = variable nucleotide and I = Inosine. The annealing temperature was between 44°C and 50°C while the distance between the position of sense primer and that of anti sense primer was 125 amino acids. Therefore, the expected exon size is 375 bp. These primers were designed using the programme Primer 3 (Steve and Skaletsky, 2000; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The oligonucleotides were synthesized by MWG Biotech AG (Ebersberg-Munich, Germany) as shown in Table 6.

3.2.5 DNA isolation and PCR

Genomic DNA was isolated from 80 mg of ground *Pelargonium* leaf using the DNeasy^{R} Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The concentration of 'Katinka' genomic DNA was determined by comparing it with standard concentrations (5, 10, 25, 50, 100 and 200 µg ml^{-1}) of λDNA (Fermentas GmbH, St. Leon-Rot, Germany) in a 1% agarose, flatbed gel electrophoresis visualised by staining with 40 µg ethidium bromide. A temperature gradient PCR was performed to optimise the annealing temperatures for the various gene-specific primers (Table 6) to minimise the number of incorrect base pairings (mismatches). This phenomenon is enhanced by low annealing temperature (Rychlick *et al.,* 1990). Additionally, if the PCR reactions are allowed to stand at room temperature for about 5 min, the sensitivity decreases by a factor of 1000 (Mullis, 1991). Therefore, in order to reduce mispriming, the time between the completion of PCR reaction probes and the starting of PCR programmes was minimised. This reduced the chance of experiencing a low primer annealing temperature, which is responsible for mismatching (Rychlick *et al.,* 1990).

Table 6: Gene specific primer pairs for β-Actin gene, ACC synthase (*PzACS1, PzACS2, PzACS3* and *PzACS4*) genes and Ethylene receptor (*PzETR1* and *PzETR2*) genes designed using the programme Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Name	**sense Primer**	**Name**	**antisense Primer**	**Annealing Temp. (^{o}C)**	**PCR Product (bp)**
***β-Actin* _for**	**5'AGATCTTTATGGAAACATTGTGCTC3'**	***β-Actin*_rev**	**5'ATCCAGACACTGTATTTCCTCTCT3'**	**53**	**150**
PzACS1s	5'AAAGGCGTGCTCTTAACCAA'3	*PzACS1as*	5'GACCCACCTCCTTCTTCCTC'3	62	721
PzACS2s	5'ACCCTCAAGGTGTCATCCAG'3	*PzACS2as*	5'CCTCTCACCTTTCCCATGAA'3	56.3	205
PzACS3a	5'CGAGCAAGCTAGTCCTCACC'3	*PzACS3as*	5'TTTTCGGGCTTGATTGTAGG'3	58.3	378
PzACS4a	5'CTGGTTCAACCTCAGCCAAT'3	*PzACS4as*	5'AGAGCTCGAACAATGGATGG'3	54.4	375
PzETR1/2a	5'GGGATGTGACGTGACAAGTG'3	*PZETR1/2as*	5'TTACCTTGTCTGCGTTGCTG'3	54.4	197
*PzETR2*a	5'CAACTGCACGAGAGTTGGAA'3	*PZETR2as*	5'GCTTTCGGTATGGTCCGTTA'3	56.3	206

β-Actin primer used as an internal reference (housekeeping gene) is bolded.

The primer pairs were constructed from the novel partial ACC synthase (*PzACS3* and *PzACS4*) genes and the other genes were obtained from the gene bank with their respective accession numbers as follows: *β-Actin* (*PoAc97*, X55751), *PzACS1* (*pGAC-1*, U17299), *PzACS2* (*GACS2*, U88971), *PzETR1* (*PhETR1*, AF141928) and *PzETR2* (*PhETR2*, AF141929).

For PCR analysis, 15 ng genomic DNA was used as a template in a 20 µl volume reaction that contained 0.03 µM of degenerate primer pair: ACSd2s and ACSdas or 1.2 µM gene-specific primer pairs (Table 6), 150 µM of each dNTP and 0.5 units of Taq DNA polymerase (Invitek GmbH, Berlin, Germany) in the 10x (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 20 mM $MgCl_2$ and 0.01% gelatin) Williams Buffer (Promega GmbH, Mannheim, Germany) as recommended by the supplier (Biometra, Göttingen, Germany). The reaction mixture was incubated in a Thermocycler (Biometra, Göttingen, Germany) and the PCR was initiated by denaturation at 94^{o}C for 2 min, followed by 40 cycles of 94^{o}C for 1 min, 53^{o}C to 62^{o}C (after optimisation of the annealing temperature) for 1 min and 72^{o}C for 2 min. The PCR included a final extension step at 72^{o}C for 30 min. and a cooling step at 4^{o}C. Each PCR reaction was repeated at least three times to reconfirm the results obtained.

3.2.6 Cloning, sequencing and sequence analysis

Orange G (30% Glycerin, 20 mM EDTA-pH 8, 0.25% Orange G and 10 ml deionised water) loading Buffer was added to the PCR products and centrifuged for 5 seconds. This mixture was then loaded into 1% agarose gels and separated in a flatbed gel electrophoresis using 1xTAE (40 mM Tris-Acetate, 1 mM EDTA, pH 8) Buffer. The gels had a capacity of either 50 ml or 150 ml and were run at 120V or 80V, respectively. Also, they were visualised by staining with 40 µg ethidium bromide and observed using a BioDocAnalyze UV trans-illuminator (Biometra, Göttingen, Germany). The sizes of amplicons were estimated by comparing them to a 100 bp-ladder DNA marker. The desirable fragments were cloned by ligation into a TA plasmid cloning vector using pCR^{R}4-TOPO^{R} TA Kit (Invitrogen, Carlsbad, CA; Appendix 15) and transformed into *Escherichia coli*.

The plasmid vector (pCR^{R}4-TOPO^{R}) was supplied linearised, the so called “activated vector”. The *Vaccinia* virus Topoisomerase I binds to duplex DNA at specific sites and cleaves phosphodiester backbone after 5’-(T/C)CCTT in one strand (Shuman, 1991) and the energy released is conserved through formation of a covalent bond between 3’ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between DNA and enzyme can subsequently be attacked only by 5’ hydroxyl of original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). The plasmid vector has two adjacent cutting surfaces and since both ends of DNA fragments are blocked by Topoisomerase, this prevents self ligation. Moreover, the vector allows direct

selection of positive recombinants via disruption of the lethal *Escherichia coli, ccd*B gene (Bernard *et al*., 1994) since the cells that contain non-recombinant vector are killed upon plating and hence there is no need for blue / white screening.

After transformation into *Escherichia coli*, 125 µl S.O.C (2% Tryptone, 0.5% Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) medium was added to the plasmid-*E. coli* mixture and shaken horizontally using a shaker at 240 rpm for 1.5 hours at 37^{o}C in order for the bacteria to multiply. Then the transformants were cultured in LB (1% Tryptone, 0.5% Yeast extract, 1% NaCl, 950 ml deionised water, pH 7.0) medium containing 150 µg µl^{-1} Ampicillin at 37^{o}C. Plasmid DNA from transformed *E. coli* was recovered using the NucleoSpin^{R} Plasmid Kit (Macherey-Nagel GmbH, Düren, Germany). Positive transformants were directly analysed using 0.2 µg T3 and 0.2 µg T7 primer pair in a PCR reaction. The nucleotide sequences for T3 and T7 were 5'-ATTAACCCTCACTAAAGGGA-3' and 5'-TAATACGACTCACTATAGGG-3' respectively. Sequencing by dideoxynucleotide method was performed commercially by MWG Biotech AG (Ebersberg-Munich, Germany). The isolated sequences were analysed using CLUSTAL W programme, European Bioinformatics Institute (EMBL; Higgens, 1994) and homology search was done using the BLUSTN programme, National Center for Biotechnology Information (NCBI; Altschul *et al*., 1997).

3.2.7 RNA isolation and RT-PCR

Total RNA was isolated from 30 mg of ground *Pelargonium* leaf treated (as described in Section 2.2) with 2 µl l^{-1} ethylene, 20 µM TDZ, 100 µM ABA or dark stored respectively for 4 days or untreated (control) using Invisorb^{R} Spin Plant RNA Mini Kit (Invitek GmbH, Berlin, Germany) following the protocols by the manufacturer. However, there was still some contaminating genomic DNA, despite following the protocol to the latter. Hence, the protocol was modified to include two DNA digestion steps. This was done by separately applying 6.7 units of DNAse I (Qiagen GmbH, Hilden, Germany) dissolved in 95 µl 10x DNase reaction (10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, pH 7.6 and kept at 25^{o}C) Buffer to the cell lysis mixture and later directly on the RNA-binding spin filter membrane. Also, mRNA comprised of about 2% of total RNA and for most applications like RT-PCR, this amount was sufficient. However, for meticulous applications such as synthesis of cDNA banks where high sensitivity is required, total RNA including Poly-A^{+}-RNA must be used. Total RNA from

'Katinka' and 'Ganymed' was determined by use of a Spectrophotometer (Biorad, California). To check for the presence of contaminating genomic DNA, total RNA was compared with standard concentrations (5, 10, 25, 50, 100 and 200 µg ml^{-1}) of λDNA (Fermentas GmbH, St. Leon-Rot, Germany) in a flatbed gel electrophoresis visualised by staining with 40 µg ethidium bromide. Stepwise (10x) dilution series were performed to obtain the required RNA concentration and then stored at -20°C for short-term or -80°C deep freezer for long-term storage, until further use. Under these conditions, no degradation of RNA is detectable for at least 1 year. Also, RNA was handled using latex hand gloves and was kept under ice when taking aliquots. Additionally, the laboratory working surfaces and equipments were cleaned with RNases degrading chemicals.

Unless otherwise stated, 1 µl of 500 pg total RNA was used as a template in a 25 µl RT-PCR reaction that contained 0.6 µM each of gene specific primer pairs (Table 6). These primers were designed such that they spanned an intron, in respective gene sequences to rule out potential genomic DNA contamination. Amplification of *ß-Actin* (housekeeping gene) by using gene-specific primers for the *Solanum tuberosum* mRNA gene (accession number X55751, Table 6) was performed as a control to ensure that equal amounts of total RNA were added to each RT-PCR reaction. In addition, 400 µM of each dNTP and 0.5 µl of QIAGEN^{R} One Step RT-PCR enzyme mix in 5 x RT-PCR buffer containing 12.5 mM $MgCl_2$ were used as recommended by the supplier (Qiagen GmbH, Hilden, Germany). This kit contained optimised components that allowed both reverse transcription and PCR amplification to take place in a single tube. Also, it was designed for use with gene-specific primers only, since use of random oligomers or oligo-dT primers would have resulted in amplification of non-specific products. Reverse transcription was done by two novel unique Omniscript and Sensiscript reverse transcriptases, which are recombinant heterodimeric enzymes expressed in *E. coli* and HotstarTaq DNA polymerase, which enabled a hot-start PCR was completely inactivated, thus it did not interfere with the reverse transcriptase reaction. The reaction mixture was incubated in a Thermocycler (Biometra, Göttingen, Germany) for 30 min at 50°C (reverse transcription), 15 min at 95°C (initial PCR activation step) followed by 35 cycles: 1 min at 94°C, 1 min at 49°C to 62°C (after optimisation of the annealing temperature for the primer pairs), 2 min at 72°C as recommended by the manufacturer (Qiagen GmbH, Hilden, Germany). The PCR included a final extension step at 72°C for 30 min. and a cooling step at 4°C. Each PCR reaction was repeated at least three times to reconfirm the results obtained. The products of PCR were separated as described for a normal PCR above.

3.3. Results

Ethylene production by *Pelargonium* cuttings

Throughout the experimental period, 20 µM TDZ increased ethylene production whereas exogenous ethylene (2 µl l^{-1}) significantly decreased ethylene production by the cuttings compared to the untreated control, respectively (Fig. 10). Storing cuttings in the dark had no significant effect on ethylene production for the first 2 days. However, on the third and fourth day after the onset of the experiment, dark storage increased ethylene production significantly. In contrast, 100 µM ABA had no effect on ethylene production by the cuttings for the first 3 days. However, on the fourth day, 100 µM ABA significantly increased ethylene production comparably to dark storage.

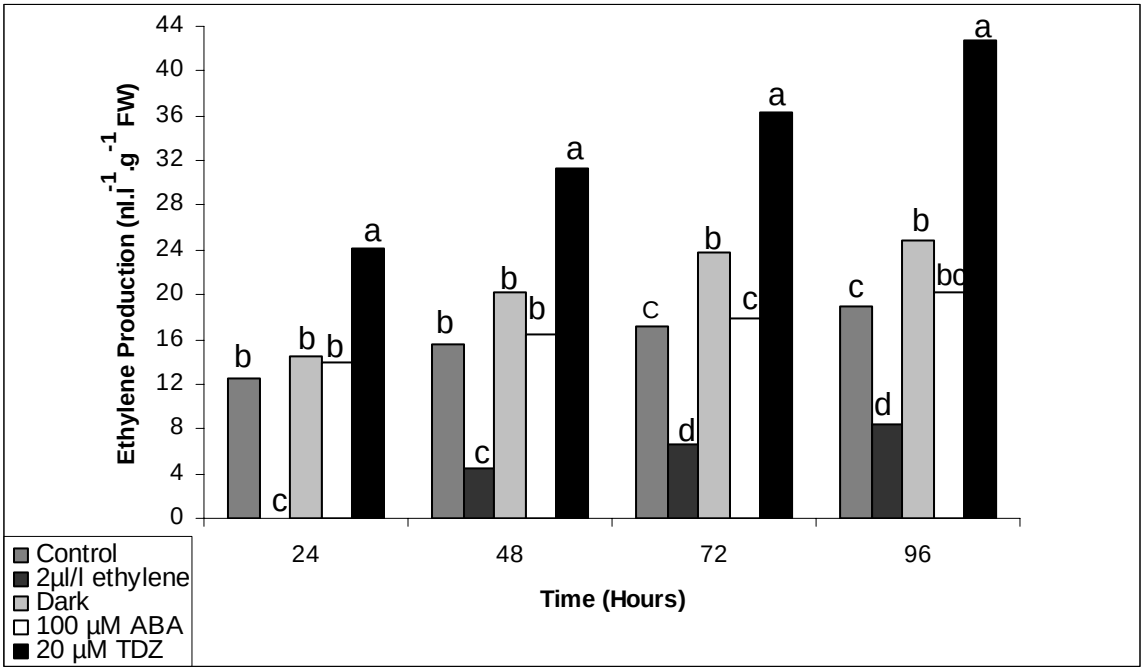

Fig. 10. Ethylene production in *Pelargonium zonale* 'Katinka' cuttings that were untreated (control) or treated with 2 µl l^{-1} C_2H_4, dark storage, 100 µM ABA and 20 µM TDZ for 4 days.

Amplification of putative ACC synthase genes

The amplification pattern for the degenerate primer pair ACSd2 using genomic DNA of *Pelargonium zonale* 'Katinka' with an annealing temperature between 44°C to 50°C resulted in three (600, 750 and 800 bp) visible fragments (Fig. 11).

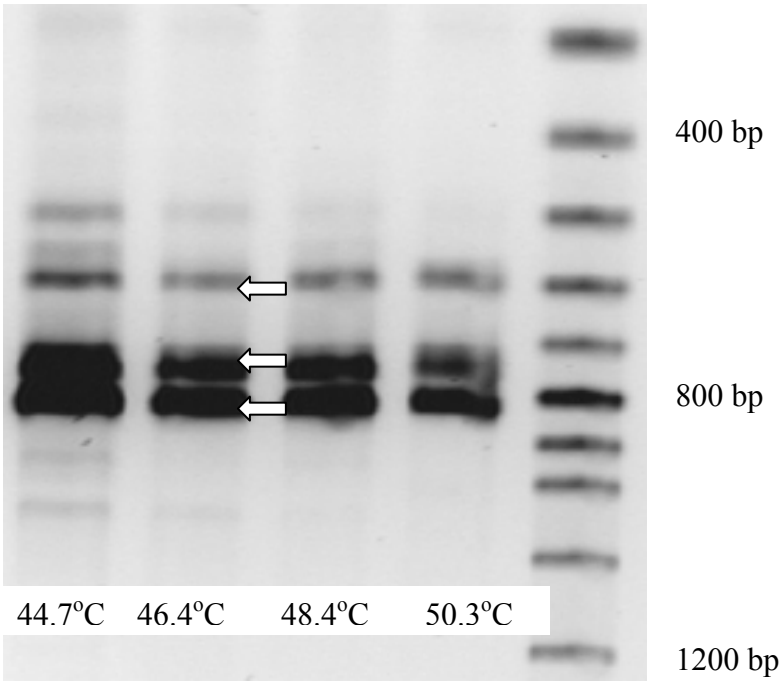

Fig. 11. Amplification patterns of putative ACC synthase genes of *Pelargonium zonale* 'Katinka' with ACSd2 primer pair and different annealing temperatures (44.7°C to 50.3°C). The 3 inner arrows indicate a 600, 750 and 800 bp fragments, respectively. Each PCR reaction was repeated at least three times to reconfirm the results obtained.

Sequence analysis

A search in the European gene bank (NCBI/BLAST) revealed that the 800 bp genomic DNA fragment sequence (Appendix 11) was identical to gGAC-2 (accession number U17230), which had been previously cloned by Wang and Arteca (1995). The 600 and 750 bp fragments were found to be novel partial putative ACC synthase genes and designated as *PzACS3* and *PzACS4*, respectively. This was because two ACC synthase genes were previously isolated in *Pelargonium x hortorum* (Wang and Arteca, 1995). Based on homology comparison with *Cucumis sativus* (*CsACS1,* AB032937*)* sequence using CLUSTAL W programme, putative exons were identified in each coding sequence of *PzACS3* and *PzACS4*

(Fig. 12). Furthermore, the binding positions for both sense and anti-sense gene specific primers were obtained for the new putative genes are shown in Fig. 12.

A. *PzACS3*

GTTCCAAGATTACCACGGGTTGCCTGCATTCAAACAAGTAAGCACTGACACATAAAACGTGTGCACTTTGACTGA
TTTAAAAAAAAAAATGTACATTTTCTAACGATATTGTACTTTTCAGGCATTGGTCGATTTTATGTCCCAAATAAGA
GGAAACAAAGTGACATTCGACCCGAGCAAGCTAGTCCTCACCGCGGGTGCCACCTCAGCCAACGAGGCTCTCATG
TTCTGCCTGGCGGATCCCGGCGAAGCCTTCCTCCTCCCCACGCCGTACTATCCAGGGTACGTATCTTTTTACGTA
CTTTTCATGTTAACAACACTATCAAGTCACATGCCAGAAACTTTATTCTAAAGAATTTTAAAAATGTACTATGTT
AACACGTCACTGATCATACTTTTGAATATTTTTCCACAGATTTGACCGAGACCTCAAGTGGCGAACTGGAGCAGA
GATCGTGCCGATCCACTGCACGAGTTCAAACGGATTTCAAATCACCGAATCGGCCCTCGAAGAAGCCTACAATCA
AGCCCGAAAACAGAACCTGAAAGTGAAGGGCGTGCTCGTGACCAACCCCTCCAATCCCCTCGGCAC

B. *PzACS4*

TTTTCCAAGATTATCACGGGTTGCCTGCTTTTAAGAACGTACGTACGTGCCATTTAATTATTTTATTGATCGACT
ATATATTTCCACAATAATTTGAAAACGCGTCTACACGTTATATATATGGGATATATAATGAGTGGATGTTGCGTA
GTTGTTTATATAATTGACATCAAACTGAGGTAGCTAGGTTGGAATGTACTTATGCAGGAAATGGTGGAGTTCTTG
TCTGCACTAAGAGGGAAGAAAGTGAAATTTGATCCAAACAACCTTGTACTCACTGCTGGTTCAACCTCAGCCAAT
GAGTCCCTCGTGTTTTGTCTAGCTCAACCTGGTGATGCTTTCCTTCTTCCCACTCCTTACTATCCCGGGTAATCA
TTTTACATATGGTCACAATTTAAAGTAGCCTTTTGATTTTAAGTTTTGGTTTTATGTGAATCACATTCAACAATG
TTATTTCCGTTATTTTTTCACATAAAACATTTTCATAATCACACTCTATTTACTGTTTTTAAGTAGTCCACATCT
CAATATACCTTTCATTCCTCACGTGTTTATTTGTGTGTGAGACAGGTTTGATAGAGATCTCAAATGGCGAACCGG
AGCTGAAATCGTTCCCATCCATTGTTCGAGCTCTAACAATTTCCGAATCACTCCTTGTGCGCTACAAGAAGCTTA
CGAGCGAGCTCAAAAACTAGGCCTAAACCCGAAAGCAGTACTGATCACCAACCCCTCCAACCCCCTCGGCAC

Fig. 12. Base pairs sequence of (A) *PzACS3*, 590 bp and (B) *PzACS4,* 745 bp. Exon; Intron; binding position for sense Primer and binding position for antisense Primer. The border between the intron and exon sequence was detected with splicing specific sequence GT and AG, respectively.

After determining the position and size of exon and intron, mRNA sequences for both genes were obtained. Conversely, *PzACS3* is 590 bp long and contains a 374 bp coding region with two introns, 83 and 133 bp long, respectively. Additionally, *PzACS4* is 745 bp in length, contains 374 bp exons and has two introns, 169 and 202 bp long (Fig. 13). Overall, the expected exon length of approximately 375 bp was confirmed in the novel putative ACC synthase genes by means of sequence analysis.

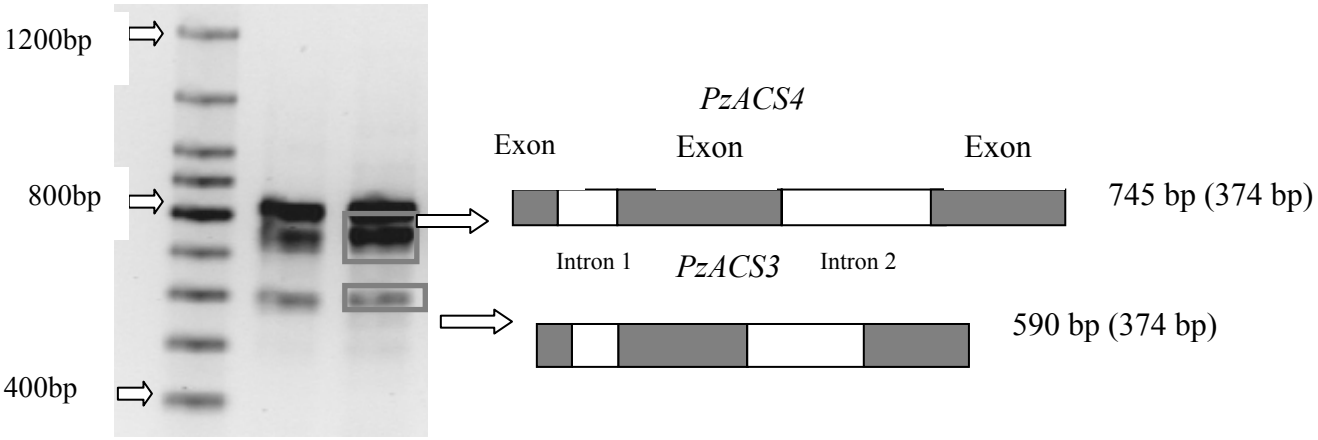

Fig. 13. Schematic representation of the new *Pelargonium zonale* putative ACC synthase genes (*PzACS3* and *PzACS4*) with respect to intron and exon sizes. In brackets is the expected exon size.

Further polypeptide sequences analysis using CLUSTAL W programme for alignment revealed that both *PzACS3* and *PzACS4* codes for 124 amino acids. These partial ACC synthase genes contain five out of eleven conserved amino acid residues common to all ACC synthases and various amino acid transferases (Fig. 14). Additionally, three out of seven conserved domains of all the ACC synthase isozymes are found in *PzACS3* and *PzACS4* (Fig. 14).

```
PzACS3          FQDYHGLPAFKQALVDFMSQIRGNKVTFDPSKLVLTAGATSANEALMFCLADPGEAFLLP 60
CsACS1          FQDYHGLPAFKKALVEFMAEIRGNKVTFEANNIVLTAGATSANETLMFCLAEAGDAFLLP 60
PzACS4          FQDYHGLPAFKNEMVEFLSALRGKKVKFDPNNLVLTAGSTSANESLVFCLAQPGDAFLLP 60
                ***********: :*:*:: :**:**.*:..::*****:*****:*:****:.*:*****

PzACS3          TPYYPGFDRDLKWRTGAEIVPIHCTSSNGFQITESALEEAYNQARKQNLKVKGVLVTNPS 120
CsACS1          TPYYPGFDRDLKWRTGVEIVPIHCTSSNGFQVTQPALEQAYQEAQARNLRVKGVLVTNPS 120
PzACS4          TPYYPGFDRDLKWRTGAEIVPIHCSSSNNFRITPCALQEAYERAQKLGLNPKAVLITNPS 120
                ****************.*******:***.*::*  **::**:.*:  .*. *.**:****

PzACS3          NPLG- 124
CsACS1          NPLGT 125
PzACS4          NPLG- 124
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Fig. 14. Alignment of deduced amino acid sequences for partial putative *PzACS3, PzACS4* and *CsACS1*. Three out of seven conserved domains of all the ACC synthase isozymes are shaded light grey. Also five out of eleven residue amino acids conserved among all the ACC synthases and various amino transferases are written in white letters. *= identical amino acid. : = two nucleotides out of the triplicate amino acid code are identical. . = one nucleotide out of the triplicate amino acid code are identical.

The nucleotide sequences of the new *PzACS3* and *PzACS4* genes were compared with sequences from other plant species using CLUSTAL W programme in order to construct a Dendrogram (Fig.15). This Dendrogram shows that *PzACS3* and *PzACS4* are closely related to each other as compared to *pGAC-1G* and *pGAC-2G* isolated earlier by Wang and Arteca (1995). Also, *PzACS3* shows a higher homology to *STAC1* from *Solanum tuberosum*.

Likewise *PzACS4* is highly homologous to *LeACS3* from tomato (Fig.15). Moreover, *pGAC-1G* and *pGAC-2G* have high sequence homology to *CsACS1* from Cucumber.

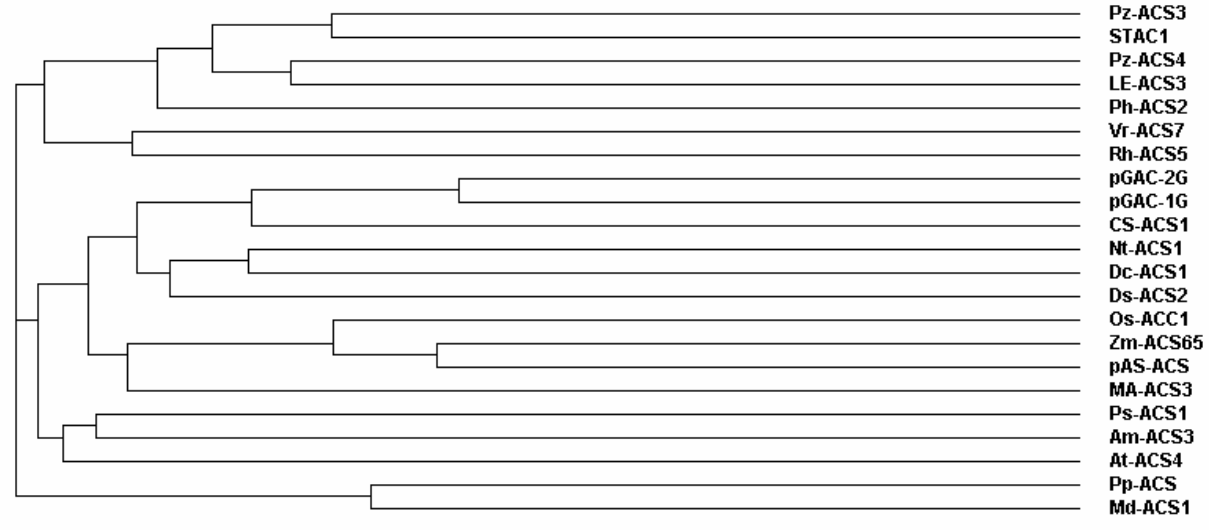

Fig.15. Dendrogram of various nucleotide sequences of ACC synthase genes from different plant species. Gene bank names and their Accession numbers: *Pz-ACS3* Pelargonium (new); *STAC1* Potato (Z27233); *Pz-ACS4* Pelargonium (new); *LE-ACS3* Tomato (L34171); *Ph-ACS2* Petunia (AF049711); *Vr-ACS7* mung Bean (AF 151961); *Rh-ACS5* Rose (AY525069); *pGAC-2G* Geranium (U17230); *pGAC-1G* Geranium (U17228); *Cs-ACS1* Cucumber (AB032937); *Nt-ACS1* Tobacco (X65982); *Dc-ACS1* Carnation (Z18952); *Ds-ACS2* Orchid (L07883); *Os-ACC1* Rice (M96673); *Zm-ACS65* Maize (AY359571); *pAS-ACS* Asparagus (AB111528); *MA-ACS3* Banana (AB021908); *Ps-ACS1* Pea (AF016458); *Am-ACS3* Snapdragon (AF083816); *At-ACS4* Arabidopsis (U23482); *Pp-ACS* Pear (AB015624); *Md-ACS1* Apple (U89156).

Expression of ACC synthase and ETR genes in tissues

To determine expression of ethylene biosynthetic genes during developmental phases, one step RT-PCR was performed on total RNA from various tissues of 'Katinka'. PCR-products with fragment sizes as predicted from their respective cDNA sequences were amplified using gene specific primer pairs. The four ACC synthase and two ETR genes were expressed in tissue-specific manner. The mRNAs of *PzACS1* were abundantly expressed in stems, leaves, flower buds, petals and pistils but were barely detectable in the roots (Fig. 16). Additionally, *PzACS2* transcripts accumulated to high levels in roots, at moderate levels in the leaves and pistils, at low levels in stems and flower buds and are almost undetectable in the petals. Interestingly, no visible *PzACS3* signals were detected in all tissues examined but only a weak band in the roots. Furthermore, *PzACS4* mRNAs were strongly expressed in the flower buds, moderately in the stems, leaves and petals whereas it was only weakly expressed in pistils and were not at all detectable in roots. The *PzETR1* transcripts accumulated to very high levels in roots and flower buds whereas only very low levels were found in stems, leaves and pistils

and were undetectable in petals. Moreover, *PzETR2* mRNAs were strongly and constitutively expressed in all plant tissues (Fig. 16).

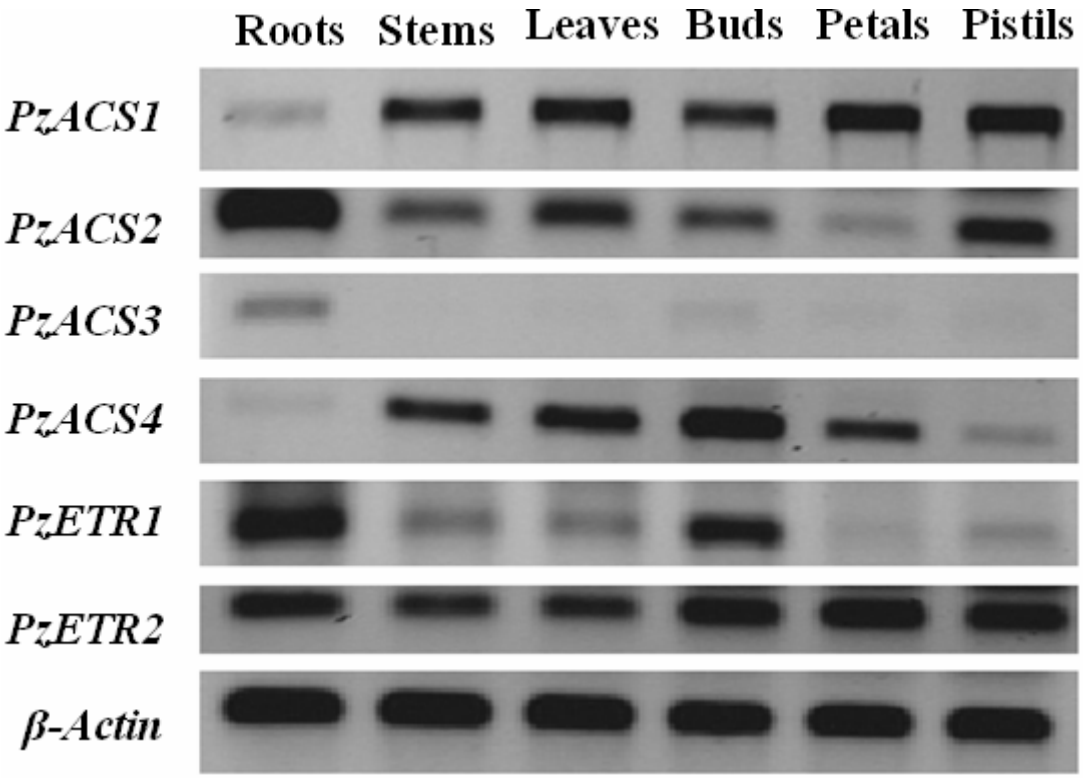

Fig.16. Expression of ACC synthase (*PzACS1, PzACS2, PzACS3, PzACS4)* and ETR (*PzETR1* and *PzETR2)* in various tissues of 'Katinka'. Roots, stems, leaves, flower buds, petals and pistils were harvested from mature plants. Petals and pistils tissues were obtained from un-pollinated flowers which had slightly opened stigmatic lobes but no signs of petal senescence. For all probes, 500 *pg* total RNA was used in each PCR reaction. β-*Actin* was used as an internal control and its uniform amplification in all tissues indicates uniform loading in each lane. The right size for each gene was detected and each PCR reaction was repeated at least three times to reconfirm the results obtained.

Expression of ACC synthase and ETR genes after treatments

One step RT-PCR was used to investigate the expression of target genes in young fully expanded leaf of 'Katinka' after application of various treatments as described in 2.2. Applying 100 µM ABA led to accumulation of high levels of *PzACS1* transcripts whereas the other treatments resulted in moderate expression levels (Fig. 17). Indeed, this ABA effect was confirmed in 'Ganymed' leaves and reconfirmed in 'Katinka' leaves too (Fig. 18A).

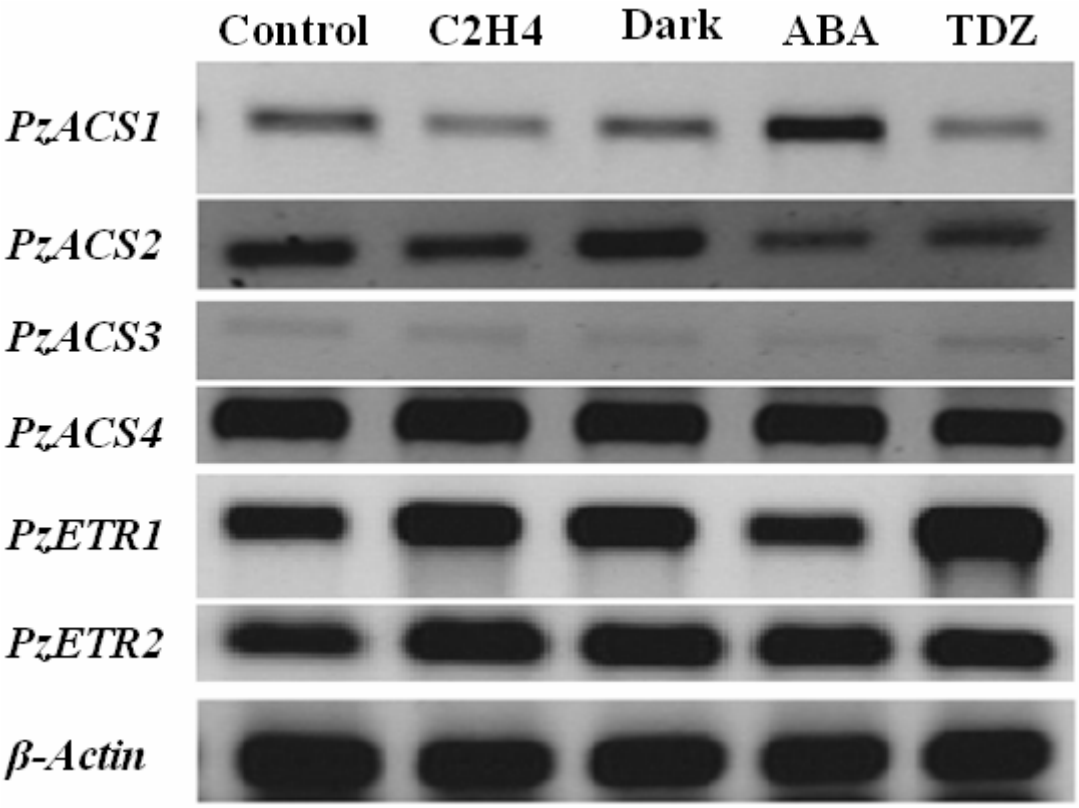

Fig.17. RT-PCR analysis of *PzACS1, PzACS2, PzACS3, PzACS4, PzETR1* and *PzETR2* transcripts accumulation in fully expanded leaf of 'Katinka' that were untreated (control) or treated with 2 µl l^{-1} C_2H_4, dark storage, 100 µM ABA and 20 µM TDZ for 4 days. For all probes, 500 *pg* total RNA was used in each PCR reaction. β-*Actin* was used as an internal control and its uniform amplification in all treatments indicates uniform loading in each lane. The right size for each gene was detected and each PCR reaction was repeated at least three times to reconfirm the results obtained.

Additionally, following dark storage of cuttings for 4 days, the levels of *PzACS2* mRNAs detected in 'Katinka' leaves were slightly increased whereas the rest of treatments induced comparable expression bands (Fig. 17). To the contrary, *PzACS2* transcripts were not detected in 'Ganymed' leaves (Fig. 18B). Furthermore, *PzACS3* transcripts were not detectable in 'Katinka' leaves (Fig. 16) and consequently its mRNAs were not detectable in the leaves irrespective of treatments (Fig. 17). Moreover, *PzACS4* and *PzETR2* mRNAs were strongly and constitutively expressed in 'Katinka' leaves for all treatments (Fig. 17). In contrast, *PzETR1* transcripts were very strongly expressed in 'Katinka' leaves in response to 20 µM TDZ application whereas the other treatments had more or less equal levels of expression (Fig. 17). Likewise treating 'Ganymed' leaves with 20 µM TDZ had similar effect (Fig. 18C). Lastly, treating 'Katinka' leaves with 2 µl l^{-1} ethylene had no effect on the accumulation of transcripts in all the genes examined (Fig. 17).

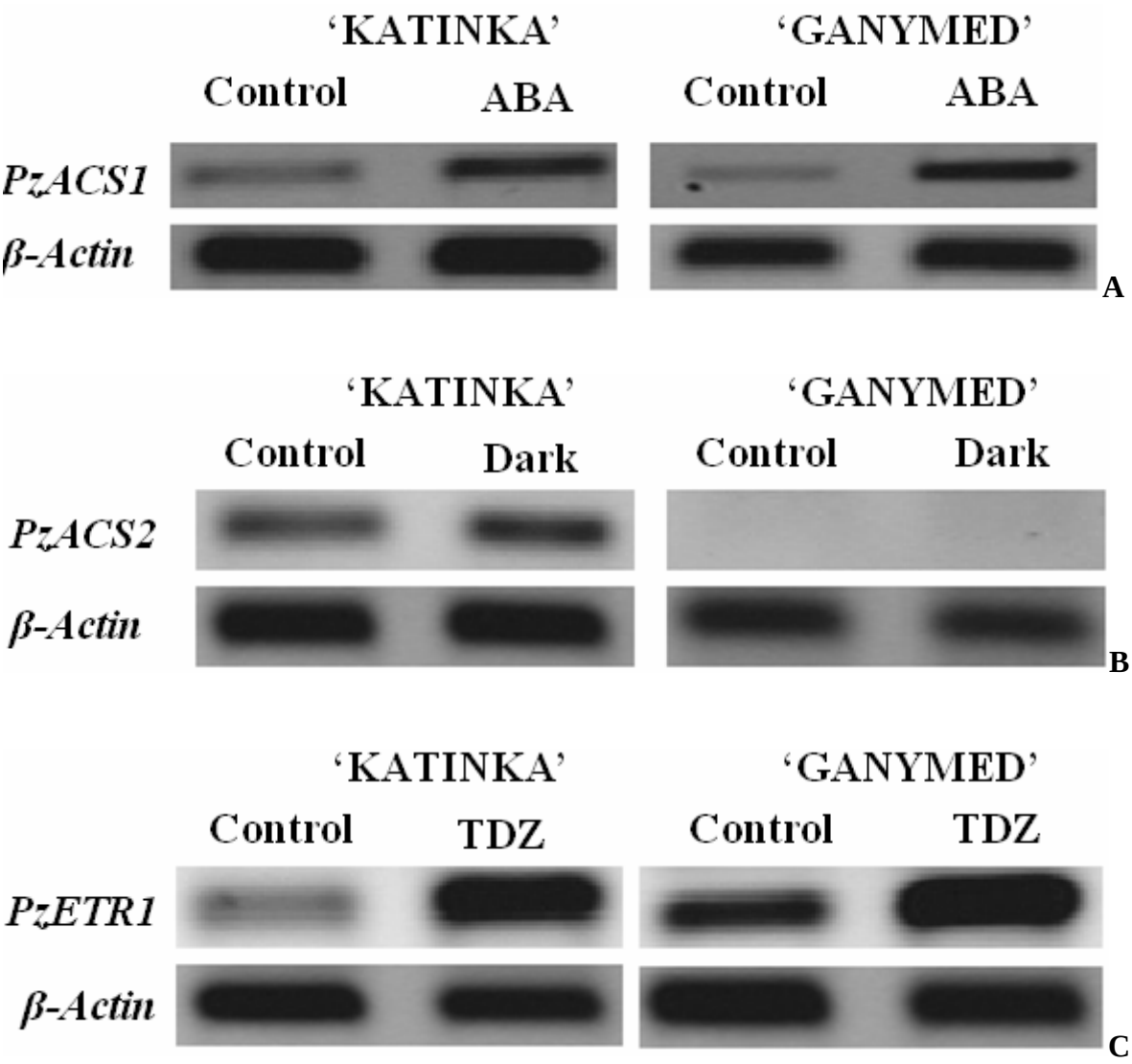

Fig.18. RT-PCR analysis of *PzACS1, PzACS2* and *PzETR1* transcripts accumulation in fully expanded leaf of 'Katinka' and 'Ganymed' after they were treated with100 μM ABA, dark storage and 20 μM TDZ or untreated (control) for 4 days. For all probes, 500 *pg* total RNA was used in each PCR reaction except for *PzETR1* where 1 pg total RNA was used. β-*Actin* was used as an internal control and its uniform amplification indicates uniform loading in each lane. The right size for each gene was detected and each PCR reaction was repeated at least three times to reconfirm the results obtained.

3.4 Discussion

3.4.1 Methods

DNeasy^{R} Plant Mini Kit used to isolate purified genomic DNA was rapid and efficient. Additionally, the advantage of PCR method against other proven methods, stems from its high sensitivity since theoretically, it can detect a single DNA molecule. However, in practice this high sensitivity is rarely achieved. Also, PCR has the disadvantage of amplifying non-specific product(s) alongside the target product(s) (Lyons, 1992). This indicates the presence of other nucleotide sequences and a possible cause for this non-specific amplification is the binding of primers to the DNA matrix that does not exactly match their complementary base sequences the so called mispriming. The plasmid vector (pCR^{R}4-TOPO^{R}) is faster (takes 5 minutes) than the conventional T4 ligase reaction and provides a highly efficient one step cloning strategy for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector for sequencing. However, it has the disadvantage of skidding at times.

Invisorb^{R} Spin Plant RNA Mini Kit used to isolate high purity total RNA had the advantage of being fast. However, it had some shortcomings as indicated in Section 3.4.7. Also, Qiagen one step RT-PCR kit was simple to use, more rapid, highly sensitive, more specific and less expensive than northern blot analysis used by other authors, with detection level of ca. 1 pg-2 µg total RNA. Also, the kit minimised the risk of contamination, as there were neither separate reverse transcriptions nor post-amplification steps. It resulted in a highly specific amplification and eliminated extension from non-specifically annealed primers and primer-dimers in the first cycle, thus ensuring highly specific and reproducible PCR. A semi-quantitative RT-PCR was used to estimate the relative abundance of transcripts in various tissues and in response to different treatments with β-*Actin* (house keeping gene) as internal control. This is because β-*Actin* is expressed at relatively constant rates in most living cells. Conversely, β-*Actin* mRNAs were strongly and constitutively expressed in all tissues including leaves of 'Katinka' and 'Ganymed' after application of different treatments. This observation not only showed the integrity of total template RNA used in all RT-PCR reactions but also confirmed uniform loading (Figs. 16, 17, 18). However, RT-PCR has a disadvantage in that, quantification is difficult because many sources of variation exist, including template concentration and amplification efficiency (Dean *et al*., 2002). For RT-PCR to be accurate and quantitative, it must be analyzed in the linear range of amplification before reaction components become limiting, which occurs after only 20 cycles. However, RT-PCR with 35

cycles was shown to closely resemble northern blot analysis, indicating relatively low template amount or amplification efficiency (Dean *et al*., 2002).

Amplification of putative ACC synthase genes

For one to amplify unknown putative genes using genomic DNA, it is important to construct a highly specific degenerate primer from the conserved exon regions of ACC synthase gene sequences. Due to the large number of known ACC synthase genes from different plants in NCBI gene bank, it was possible to design appropriate degenerate primer pairs using ACC synthases from *Cucumis sativus* L as an example, and the subsequent sequence analysis of the amplicons was simple (Rottmann *et al*., 1991). The specific degenerate primer used made it possible to make amplification from less genomic DNA templates. Conversely, each of the 3 fragments cloned represented a putative ACC synthase gene and the difference in the size of amplicons resulted possibly from the difference in their intron numbers and their size (Fig. 11). Additionally, the degenerate primer pairs exhibited variability in intron size between the sense and anti-sense primer binding sites within the ACC synthase gene family (Mibus, 2003). The rationale behind this approach is to amplify many putative ACC synthase genes with only one PCR reaction and it is also possible to apply it within other gene families.

Most of the cloned ACC synthase genes were amplified from cDNA. These cDNAs were generated from mRNA, hence there was very little or no difference at all in size among members of one gene family (Mibus and Serek, 2004). Therefore, separation and differentiation of different ACC synthase genes was difficult. However, cDNA (no introns) amplified products were smaller than those from genomic DNA (with introns). The size difference in products was used to detect presence of contaminating genomic DNA in the template RNA, because the length of genomic PCR products was approximately 100 bp longer than cDNA products (Caelers *et al*., 2004). Moreover, where only mRNA sequence was known, a primer annealing site with more than 300-400 bp apart were chosen as in *PzACS2* (720 bp). It was most likely that fragments of this size from eukaryotic DNA contained a splice junction that was used to detect genomic DNA contamination. Another disadvantage of using cDNA as template DNA was the need to have prior knowledge about the tissue or developmental stage at which the gene is expressed. Furthermore, construction and screening of a cDNA or genomic bank with heterologous probes from other species is a more effective but both time consuming and cost intensive method (Mibus and Serek, 2004).

Consequently, to clone genes of different plant species within a short time, the construction of a cDNA or genomic bank of each plant species is not feasible. In general, it is easier to clone genes from cDNA than genomic DNA because the number of templates is much higher at the mRNA level compared to cDNA. Furthermore, a potential problem with genomic DNA occurs if the gene specific primers are based on protein or cDNA alignments and one of the primers span an exon-intron junction (Mibus and Serek, 2004).

The isolation of two new partial ACC synthase genes in this study (Fig. 13) indicates presence of a multigene family in *Pelargonium* just like in other plants. In *Arabidopsis*, many gene products encode isoforms of the same polypeptide (The Arabidopsis Genome Initiative, 2000). The biological significance of multigene families and of ACC synthase gene family in particular is unknown. However, it has been postulated that tissue-specific expression of a particular ACC synthase isozyme satisfies the biochemical environment of the cells and tissues in which each isozyme is expressed (Rottmann *et al*., 1991). Consequently, this concept enhances the physiological fine-tuning of the cell and demands that the enzymatic properties of each isozyme be distinct (Graur and Li, 2000). Furthermore, ACC synthase polymorphism may reflect the evolution of a family of proteins with different enzymatic properties (K_m, pI etc.) to effectively utilize S-adenosyl-methionine in different tissues during plant growth and development or under different stress conditions (Rottmann *et al*., 1991). Indeed, *PzACS3* was only expressed in roots whereas it was not detected in leaves (Fig. 16), which supports the views of Rottmann *et al*. (1991) and seems to hold true for the new *Pelargonium* genes.

3.4.2. Results

Ethylene production

Ethylene elicits different responses in a variety of plant tissues (Abeles *et al*., 1992). Therefore, the ability of a given tissue to perceive ethylene becomes an important aspect of plant development. Exogenous application of ethylene inhibited ethylene evolution by *Pelargonium* cuttings ‘Katinka’ (Fig. 10), which are mainly comprised of vegetative tissues (leaves and stems). This was expected since Philosoph-Hadas *et al*. (1985) demonstrated auto-inhibition of ethylene production in tobacco leaf discs. Vegetative tissues are less sensitive to exogenous ethylene application as opposed to floral tissues (Abeles *et al*., 1992). Moreover,

both positive and negative feedback regulation of ethylene biosynthesis have been reported in different species (Nakatsuka *et al*., 1998). For instance in carnation, ethylene production in the leaves was slightly inhibited by treatment with ethylene (Henskens *et al*., 1994).

Various forms of stresses induce ACC and ethylene production in a variety of plant species and are thought to be responsible for inducing leaf senescence (Abeles *et al*., 1992). Dark storage, a form of stress increased ethylene evolution in 'Katinka', 3 and 4 days after the beginning of the experiment (Fig. 10). Additionally, dark storage positively regulated the accumulation of *PzACS2* transcripts (Fig. 17). This may be responsible for the increased ethylene evolution that led to the observed leaf senescence manifest as leaf yellowing (Fig. 2). Furthermore, stress-induced ethylene production is typically controlled by accelerating the conversion of S-AdoMet to ACC (Kende, 1993).

Applying 100 µM ABA increased ethylene production comparably to dark storage on the fourth day (Fig. 10) and up-regulated *PzACS1* transcripts in 'Katinka' (Fig. 17). Taken together, these results indicate that ABA hastens ethylene production by increasing the expression of *PzACS1*. Therefore, it is this ABA-induced ethylene that made the cuttings to become chlorotic (Fig. 2). Furthermore, Hanley and Bramlage (1989) confirmed using enzyme-linked immunosorbent measurements that higher ABA levels paralleled ethylene increase and onset of irreversible wilting in carnation petals. However, while ABA has been shown to stimulate ethylene evolution in a number of species, it also inhibits senescence and water stress-induced ethylene in others (Riov *et al*., 1990).

TDZ (20 µM) markedly increased ethylene production throughout the study period (Fig. 10). A plausible explanation is that TDZ bound into ethylene receptors, in the same way like *AHK4* gene (Yamada *et al.,* 2001), thus preventing ethylene from binding to the same receptors. This is because *AtETR1* is highly homologous to *AHK4* gene, which acts as a primary cytokinin receptor that directly binds cytokinins including TDZ (Yamada *et al.,* 2001). Therefore, blocking the ethylene receptors would inhibit the down-regulating action of ethylene. Conversely, since cuttings could not detect presence of ethylene, they responded by possibly inducing a positive ethylene feedback mechanism leading to observed increase in ethylene production. Similarly, enhanced ethylene production in response to MCP application was recently reported in *Pelargonium* (Kadner and Druege, 2004). Furthermore, TDZ increased ethylene production in hypocotyl cultures of geranium (Hutchinson *et al*., 1997),

mung bean hypocotyl segments (Suttle, 1984) and suspension cultures (Yip and Yang, 1986). Moreover, cytokinins at low doses (0.5-10 µM BA) have been shown to stimulate ethylene production in etiolated *Arabidopsis* seedlings and to induce morphological (triple response) changes typical of ethylene (Cary *et al*., 1995).

Gene expression in tissues

Generally, *PzACS1*, *PzACS2* and *PzACS4* were expressed in most of the tissues examined except *PzACS2*, which was not detectable in 'Ganymed' leaves (Fig. 18B). Therefore, the observed difference in the tissue-specific gene expression pattern from other reports is attributable to genotypic differences among the cultivars and different analysis methods used. Also, *PzETR2* was constitutively expressed. Theses results differ with those of Wang and Arteca (1995) in that, *PzACS1* was expressed in all tissues except roots in this study while Wang and Arteca (1995) using northern hybridization were unable to detect *PzACS1* in any plant part. Also, Hilioti *et al*. (2000) supports these findings because they found using northern hybridization, specific expression of *GAC-1* in stigma and style tissues and observed that the abundance of *GAC-1* in vegetative tissues (such as leaves, roots and stems) was too low to be detected in 30 µg total RNA (Hilioti *et al*., 2000). Hilioti *et al*. (2000) attributed the observed differences to the fact that GAC-1 expression is difficult to detect in whole pistil extracts. This is because it is expressed only in stigma and style, which represent 5% of the total fresh weight of pistil. Additionally, *PzACS2* mRNAs were expressed in all tissues albeit weakly in petals (Fig. 16). Similar results were obtained by Clark *et al*. (1997) in that *GACS2* was not expressed in leaves as shown in 'Ganymed' (Fig. 18) but it was expressed in pistils, roots and stems whereas it was barely detectable in petals. However, Wang and Arteca (1995) reported that *GAC-2* transcripts were expressed to a lesser degree in fully expanded leaves or roots but was undetectable in old leaves and floret buds. Moreover, while *PhETR1* was equally expressed in all tissues (Fig. 16), Dervinis *et al*. (2000), using northern hybridization found highest, lowest and moderate expression in pistils, roots and rest of the examined tissues, respectively. *PzACS2* transcripts were not expressed in 'Ganymed' leaves unlike in 'Katinka', which is a clear evidence for genotypic difference. Moreover, while the other authors used northern hybridization method, RT-PCR method used in this study was highly sensitive.

The distinct tissue-specific gene expression pattern observed among members of ethylene biosynthetic pathway families suggests that they play crucial role(s) in these tissues. For instance, the new *PzACS3* gene was exclusively expressed in a vegetative tissue (root) while *PzACS1* and *PzACS4* were expressed both in vegetative and reproductive tissues but later in the development stage. Also, *PzETR1* was strongly expressed in roots and flowers buds. In contrast, *PzACS2* and *PzETR2* were expressed throughout all the developmental stages of 'Katinka'. Zhou *et al*. (1996) found mRNAs for an *ETR1* homologue in tomato were constitutively expressed in both vegetative and reproductive tissues. Additionally, ACC synthase enzymes were found to be spatially and temporally regulated and were controlled by internal signals (Nakatsuka *et al*., 1998). This leads to the conclusion that differential expression of individual members of the gene family may lead to differential sensitivities of tissues to ethylene throughout plant growth and development (Rottmann *et al.,* 1991; Yamagami *et al*., 2003).

The abundant expression of *PzACS1* in almost all tissues except roots suggests it plays a vital role in ethylene biosynthesis (Wang and Arteca, 1995) that occurs in all cells. After biosynthesis, ethylene moves freely throughout the plant tissues (Yang and Hoffman, 1984). Additionally, since *PzACS1* is strongly expressed in pistils, it seems likely that it plays a role in pollination-induced ethylene production. Indeed, the findings of Hilioti *et al*. (2000) support current study in that specific expression of *GAC-1* cDNA was detected 2 h after pollination in the stigma and style tissues and correlated very well with the peak in ethylene production and pattern of pollen tube penetration into the style (Clark *et al*., 1997). This suggests one of the functions of *PzACS1* maybe related to preparation for and/ or facilitation of pollination. Moreover, the initial signalling should originate with live pollen since neither heat-killed pollen nor mock pollination with sand, induced post-pollination responses (Clark *et al*., 1997). Conversely, the degree of response was modulated by pollen load, since increasing the number of pollinated lobes increased both ethylene production and petal abscission (Hilioti *et al*., 2000).

An alternative explanation is possible since *PzACS1* and *PzACS2* transcripts accumulated in pistils. Consequently, it seems likely that wounding resulting from excision of pistils from the flowers could induce their transcriptional regulation. Ethylene biosynthesis is known to be stimulated by wounding, most likely by induction of ACC synthase activity (Watanabe *et al*., 2001). Wound-induced ACC synthase genes were differentially expressed in carnation

flowers (Park *et al*., 1992). Furthermore, the stigmatic recognition of pollen and tissue damage associated with stigma penetration and pollen tube growth leads to increased ethylene production (Abeles *et al*., 1992).

The fact that *PzACS3* transcripts are specifically expressed in the roots of 'Katinka' indicates that one of its functions could be involvement in the ethylene biosynthesis that takes place in the roots of *Pelargonium*. Indeed, ethylene is involved in adventitious root formation of transgenic tomato cuttings (Clark *et al*., 1999), root hair development and nodulation (Wang *et al*., 2002). Therefore, in plant organs other than roots, other ACC synthase genes may function as vital components of ethylene biosynthesis. In this regard, *PzACS1* and *PzACS4* are the best candidates. However, *PzACS4* seems the most likely gene because it is expressed in several shoot organs (stems, leaves and petals) and its polypeptide sequence is highly homologues to that of *PzACS3*. A similar hypothesis was advanced by Ueguchi *et al.* (2001b) with respect to histidine kinase 4 (AHK4) genes.

Transcripts of *PzACS4* were strongly expressed in the flower buds and moderately in the petals. Additionally, *PzACS1* mRNAs were also abundant in petals. Pollination is known to evoke rapid non-autocatalytic ethylene evolution followed by abscission of turgid petals (Clark *et al*., 1997; Hilioti *et al*., 2000). Therefore, it seems possible that both *PzACS4* and *PzACS1* play a role in ethylene produced at the bases of petals which leads to their abscission. Indeed, despite very low ACC oxidase activity, it was shown that pollination induced ACC synthase activity at the base of petals (Hilioti *et al*., 2000). In contrast, low expression of *PzACS4* transcripts in the pistil implies that it is partially not involved in the pollination non-autocatalytic ethylene production that occurs specifically in the stigma and style (Hilioti *et al*., 2000).

Expression studies revealed that *PzETR2* mRNAs were strongly and constitutively expressed in all plant tissues. This coupled with distinct expression patterns of ACC synthase genes would allow ethylene to be synthesised and perceived in a tissue-specific manner, in order to mediate various biological responses. It was observed that during tomato fruit ripening, there is up-regulation of *NR* (Lashbrook *et al.,* 1998) and the same is true for *Nt-ERS1* during tobacco development (Terajima *et al*., 2001). Therefore, it seems likely that various ethylene biosynthetic genes have been tailored in the course of evolution, so that they are appropriate

for the specific metabolic and perception roles they sub-serve in the particular intracellular environment of the tissues in which they are found (Yamagami *et al*., 2003).

Transcripts of *PzETR1* accumulated to very high levels in the roots and flower buds (Fig. 16). This suggests that *PzETR1* is expressed early during root development and it is could be involved in making the stem tissues of *Pelargonium* cuttings to be less sensitive to ethylene. This would have negative effect especially during adventitious root formation as previously reported in Petunia cuttings (Clark *et al*., 1999). On the other hand, *PzETR1* expression in flower buds indicates that it is expressed early during flower development. Also, it is likely that *PzETR1* could be expressed later during flower development and senescence in at least one of flower tissues (sepals, pistils or stamens). The former idea is supported by results of Dervinis *et al*. (2000), whereby accumulation of *PzETR1* and *PzETR2* mRNAs occurred early in pistil development, when detectable amounts of both transcripts were expressed at tight bud stage (Dervinis *et al*., 2000) and the florets were insensitive to exogenous ethylene (Evensen, 1991). Furthermore, mRNA levels of both *PzETR1* and *PzETR2* remained constant throughout pistil development with a slight increase in post-pollination receptive pistils in geranium (Dervinis *et al*., 2000). This is also consistent with the findings of Lashbrook *et al.* (1998) with tomato *LeETR1*, *LeETR2* and *Nr*, which did not increase more than three fold during floral development. Additionally, genomic southern analysis revealed that both *PzETR1* and *PzETR2* are members of same gene family (Dervinis *et al*., 2000) but it is not known why ethylene receptors exist like so. However, Lashbrook *et al.* (1998) suggested that individual ethylene receptors maintain a distinct functional identity via the capacity to respond differentially to developmental cues.

Expression of genes in leaves after treatments

Exogenous ethylene slightly down regulated expression of *PzACS1* mRNAs but had no effect on accumulation of *ETR1* genes investigated. These results indicate that *Pelargonium* leaves do not respond to ethylene by producing more ethylene. However, since exogenous ethylene lead to inhibition of ethylene production (Fig. 10), it implies that ethylene possibly inhibited the action of *ACC* oxidase or another as yet to be determined *ACC* synthase. Similar conclusions were independently arrived at by Wang and Arteca, (1995), Clark *et al*. (1997) and Dervinis *et al*. (2000). Ethylene production in carnation leaves was slightly inhibited by ethylene application. Consequently, no *ACC* synthase transcripts were detected (Henskens *et*

al., 1994). Additionally, *LeACS2* and *LeACS4* were positively regulated whereas *LeACS6* was negatively regulated by ethylene synthesised during tomato fruit ripening (Nakatsuka *et al*., 1998). Further support for this view comes from Kadner and Druege (2004) whose results strongly indicated a negative ethylene biosynthesis feedback control mechanisms in zonal *Pelargonium* cuttings. Therefore, this study suggests that exogenous ethylene does not transcriptionally activate the ethylene biosynthetic pathway genes during leaf senescence. Moreover, cycloheximide completely blocked pollination-induced (Hilioti *et al*., 2000) and ethylene-induced (Evensen *et al*., 1993) petal abscission in geranium while ethylene production was unaffected suggesting protein synthesis is required for leaf and petal abscission (Abeles *et al*., 1971; Ten Cate *et al*., 1975). This is evidence that post-transcriptional control of ethylene biosynthesis occurs primarily at the level of *ACC* synthase (Hilioti *et al*., 2000).

Applying 100 µM ABA increased *PzACS1* transcripts in 'Katinka' leaves and correlated positively with ethylene production (Fig.10). However, 100 µM ABA had no influence on other genes. It follows that ABA was involved in ethylene biosynthesis of *Pelargonium* cuttings through enhancement of *PzACS1* activities that led to the observed increase in ethylene production. Moreover, it was shown that ABA has a direct effect on ethylene biosynthesis in that it stimulated ethylene production in citrus leaf tissues directly through enhancement of ACC synthesis (Sagee *et al*., 1980; Riov *et al*., 1990) which supports the results of this study. Also, Müller *et al*. (2000b) speculated that ABA either increased rose tissues sensitivity to ethylene just as it did in cut carnations (Ronen and Mayak, 1981) or participated directly or indirectly in the ethylene signal transduction pathway.

Dark storage slightly increased expression of *PzACS2* but had no effect on the rest of studied genes. This is an evidence for a stress-induced ethylene evolution since it correlated positively with ethylene production (Fig 10) and could have resulted from increased activities of *ACC* synthase enzyme (Woodson *et al*., 1993). Generally, increases of *ACC* synthase activity are typically associated with increased levels of *ACC* synthase message (Peck and Kende, 1998b), suggesting that the expression of ACC synthase is the major target of regulation (Kende, 1993). Moreover, expression of ACC synthase correlated positively with ethylene levels in senescing *Pelargonium* cuttings (Fig. 10) and rose flower petals (Wang *et al*., 2004). Therefore, darkness increased *PzACS2* transcripts accumulation that led to enhanced ethylene production which was responsible for leaf yellowing. Furthermore, storing *Pelargonium*

cuttings shortly after harvest leads to accumulation of wound-ethylene in the packing material (Kadner *et al*., 2000), which promotes senescence as evidenced by leaf chlorosis (Schatz, 1982; Roberts *et al.,* 1985).

PzETR1 transcripts but not of other genes were strongly induced by exogenous application of 20 µM TDZ in *Pelargonium* leaves. Additionally, *PzETR1* mRNAs were strongly expressed in 'Katinka' roots (Fig. 16). Also, TDZ increased ethylene production in *Pelargonium* cuttings 'Katinka' (Fig. 10). In *Arabidopsis*, there are at least 11 sensor His-kinases (The Arabidopsis Genome Initiative 2000). Except for the *ETR1*-family of ethylene sensors, no external stimulus has yet been assigned convincingly for the other His-kinases (Suzuki *et al*., 2001). Moreover, the fact that *AHK4* is specifically expressed in the roots of *Arabidopsis* (Ueguchi *et al*., 2001a) and *AHK4* cDNA confers cytokinin responsiveness on yeast cells (Ueguchi *et al*., 2001b) strongly supports the idea that *AHK4* functions as a primary receptor, which directly and specifically binds a variety of natural and synthetic cytokinins including TDZ (Ueguchi *et al*., 2001b; Yamada *et al*., 2001) causing activation of His-kinase of *AHK4* (Suzuki *et al*., 2001). Additionally, *AHK4* is capable of interacting with *Arabidopsis* Hpt factors (AHPs) through phosphorelay (Suzuki *et al*., 2001). This is in contrast to the case of *ETR1*, in that binding of ethylene to the receptor is assumed to result in inactivation of its kinase activity in *Arabidopsis* leaves (Hua and Meyerowitz, 1998), Additionally, exogenous ethylene induced expression of *LeETR3* in tomato leaves (Wilkinson *et al*., 1995), *ERS2* in carnation (Shibuya *et al*., 2002) and *RhETR2* and *RhETR3* in miniature rose flowers (Müller *et al*., 2000b) whereas endogenous ethylene induced expression of *ERS1* in *Delphinium* (Kuroda *et al.*, 2003). Also, exposure of *Arabidopsis* plants to salt (NaCl) or osmotic stress reduced expression of *ETR1* (Zhao and Schaller, 2004). However, the foregoing explanation seems not tenable in the current study, since ethylene had no effect on induction of both ACC synthase and *PzETR1* genes in *Pelargonium* leaves.

Interestingly*, AtETR1* is highly homologous to *AHK4* gene in its entire amino acid sequences in *Arabidopsis* including N-terminal extensions as well as His-kinase and receiver domains (Yamada *et al.,* 2001). Ethylene receptor, *AtETR1* contains N-terminal transmembrane domains that encompass the ethylene-binding site. The *AHK4* gene acts as a primary cytokinin receptor that directly binds cytokinins (both natural and synthetic) including TDZ and presumably functions as His-kinase since it is involved in His-Asp phosphorelay signalling (Yamada *et al.,* 2001). Likewise, *AHK4* locus lies within the vascular tissue of *Arabidopsis* roots just as in *Pelargonium* (Fig. 16). Additionally, both a loss-of-function

ahk4-1 mutant and a wooden leg (*wol*) mutant in *Arabidopsis* resulted in no visible inhibition of root growth, greening or shoot induction of calli (Ueguchi *et al*., 2001b) and in reduced cell number and exclusive xylem differentiation, respectively (Mähönen *et al*., 2000). Furthermore, the root meristems are the sites of cytokinin biosynthesis (Itai and Birnbaum, 1996). This observation is consistent with a potential cross-talk between ethylene and cytokinin signalling (Ciardi and Klee, 2001). Conversely it is speculated that there is a link between cytokinin signalling and vascular morphogenesis in *Pelargonium* roots. Moreover, based on genetic and biochemical experiments in tomato (Klee, 2002) and *Arabidopsis* (Tieman *et al*., 2000), it is generally accepted that most members of ethylene receptors act as negative ethylene regulators of downstream responses, that is, in the absence of ethylene, receptors actively suppress expression of ethylene responsive genes (Klee, 2002).

Consistent with this idea, a plausible explanation of the observed TDZ effect would be that it binds to the membrane-localized ethylene receptor(s) thus blocking basal ethylene from binding to the same. Another possibility is that a cross-talk between TDZ and ethylene could have taken place via mitogen-activated protein kinase (MAPK) cascade in the ethylene signal transduction. This occurred possibly between *CTR* and *EIN2* (in the cytoplasm) or at the level of the transcription factors (in the nucleus) (Kieber *et al*., 1993; Ueguchi *et al*., 2001b). Therefore, since the cuttings could not detect ethylene, they possibly responded by inducing a positive feedback mechanism leading to observed increase in ethylene production (Fig. 10). *Pelargonium* cuttings induced mechanisms that led to generation of more ethylene receptors (Fig. 17) in an attempt to bind ethylene thus making the leaves to become insensitive to ethylene. Furthermore, ethylene sensitivity in plants is dependent on the amount of receptor protein present (Klee, 2002), in that more ethylene receptor reduces sensitivity. It is hereby concluded that that retardation in the onset of leaf senescence after TDZ application was responsible for making the *Pelargonium* leaves to remain green for a longer time.

3.5 Conclusion

Novel partial putative *PzACS3* and *PzACS4* genes were isolated, characterised and found to be expressed in a tissue-specific fashion. Also, the expression of the transcripts of novel genes were not influenced by any of the treatments applied suggesting they may be developmentally regulated. Moreover, the current study provided evidence, for the first time that *PzETR1* is strongly induced by TDZ in addition to being developmentally regulated. Since, the same level of TDZ retarded the onset of leaf senescence, it seems likely that TDZ reduces sensitivity of *Pelargonium* leaves to ethylene exposure by increasing the amount of ethylene receptors. However, the reverse was true for *PzETR2* in that it was strongly and constitutively expressed in all plant tissues irrespective of developmental stage or treatment applied. Additionally, *PzACS1* transcripts were developmentally regulated and induced by exogenous ABA application and in deed ABA increased ethylene production in *Pelargonium* leaves. Furthermore, transcripts of *PzACS2* were developmentally controlled and were also induced by the presence of darkness in storage, which is a form of stress, exemplifying an evidence of stress-induced ethylene evolution. Since both ethylene biosynthesis and perception contribute to the regulation of ethylene response in plants via each plants' ethylene threshold concentration (Wilkinson *et al*., 1995). These results suggest that the response of *Pelargonium* cuttings to both biotic and abiotic factors in the course of growth and development, especially during leaf senescence are modulated by changes in the expression levels of ACC synthase and ethylene receptor genes.

Summary

The effects of treatments with TDZ, ethylene and ABA or dark storage during leaf senescence process were studied to possibly come up with a potential commercial approach for improving post harvest quality of *Pelargonium* cuttings. TDZ delayed the onset of leaf senescence because leaves remained green. Additionally, Ferrante *et al*. (2002a, 2002b) found TDZ prevented leaf yellowing in *Alstroemeria* cut flowers and cut chrysanthemum 'Regan bianco' by inhibiting chlorophyll degradation. In contrast, ethylene, ABA and dark storage accelerated leaf senescence since leaves turned yellow. Promotion of leaf senescence by ABA and dark storage was indirectly through increased ethylene production after 4 days (Fig. 10). Moreover, ethylene has been shown to induce premature leaf yellowing in *Pelargonium* as a result of accelerated chlorophyll degradation (Purer and Mayak, 1989; Serek *et al.,* 1998).

Ethylene increased rooting proportion of cuttings while TDZ severely inhibited rooting. Additionally, ethylene, ABA and dark storage reduced growth of roots. This was shown as reduced number of roots per cutting, reduced root length, root water content, root fresh and dry weights. Furthermore, applying 4 µl l^{-1} IBA in rooting solutions induced maximal rooting proportion in 'Ganymed', increased numbers and length of roots, fresh weight and root dry matter (%) accumulation in all cultivars. The fact that 1-MCP (ethylene action inhibitor) reduced rooting in *Pelargonium* cuttings (Serek *et al*. (1998) support the view that ethylene is involved in adventitious rooting. ABA and dark storage reduced growth of roots indirectly through increased ethylene production (Fig. 10). Ethylene promotes rooting over a narrow range of concentrations (Clark *et al*., 1999).

Root inhibitory effects of TDZ may partly be attributed to TDZ being a cytokinin, which is very stable in *Pelargonium* leaves as shown by Mok and Mok (1985). The concentration-dependent effects of ethylene (Ma *et al*., 1998) and difference in sensitivity of experimental plant organs to ethylene at different stages of development (Clark *et al.,* 1999) may account for the conflicting rooting results in literature. Moreover, root promoting effects of IBA could be via enhanced synthesis of hydrolytic enzymes, which induces hydrolysis of starch and other nutrients that are directly involved in initiation of roots and formation of vascular tissue (Kracke *et al*., 1981). Also, auxins have been shown to induce ethylene synthesis in many plant species and tissues (Kawase, 1971). Therefore, current results suggest that auxin-induced ethylene may account for the observed root promoting effect of IBA. Overall,

Koukourikou-Petridou and Bangerth, (1997) concluded that adventitious root formation is a complex intrinsic balance between auxins and cytokinins.

Two (*GAC-1* and *GAC-2*) members of the putative ethylene ACC synthase gene family were isolated in *Pelargonium* (Wang and Arteca, 1995). In this study, two novel partial putative additional members of *ACC* synthase family were cloned, characterised and designated as *PzACS3* and *PzACS4*. These novel *ACC* synthase genes and other *ACC* synthase or ethylene receptor (*ETR1*) genes were expressed in a tissue-specific fashion suggesting they have different roles in ethylene biosynthesis and signaling. The biological significance of a multigene ACC synthase gene family is unknown. However, Rottmann *et al*., (1991) postulated that tissue-specific expression of a particular ACC synthase isozyme satisfies the biochemical environment of the cells and tissues in which each isozyme is expressed. Additionally, ACC synthase enzymes were found to be spatially and temporally regulated and were controlled by internal signals (Nakatsuka *et al*., 1998).

Gene specific primers for the new, other ACC synthase and ethylene receptor (ETR) genes were expressed in a treatment-specific fashion and correlated positively with ethylene production by cuttings after 4 days. However, ethylene slightly down regulated the accumulation of mRNAs for *PzACS1* gene, implying it deactivates this gene during leaf senescence. Furthermore, in vegetative tissues, ethylene leads to negative feedback regulation of its biosynthesis, which explains observed ethylene evolution inhibitory effects of 2 $\mu l\ l^{-1}$ ethylene. Also, ABA and dark storage increased *PzACS1* and *PzACS2* mRNAs, respectively which is an evidence for ABA and stress-induced ethylene evolution that possibly resulted from increased activities of enzymes ACC synthase (Woodson *et al*., 1993). For the first time, TDZ was shown to strongly induce expression of *PzETR1* in the current study. These (physiological and molecular) results suggest leaf yellowing in *Pelargonium* cuttings was due to either stress-induced ethylene that occurs after dark storage or ABA treatment and TDZ was able to antagonise their deleterious effects, possibly by increasing the amount of ethylene receptors via up-regulation of *PzETR1* transcripts, thus reducing sensitivity of leaves to ethylene with the concomitant beneficial effect of delaying the onset of senescence. Also, the yet unidentified role of TDZ in ethylene biosynthesis and perception, possibly as an inhibitor of ethylene perception through over-expression of ethylene receptor genes may partly account for the difficulty in rooting of TDZ treated that was effectively offset by application of IBA in rooting solutions.

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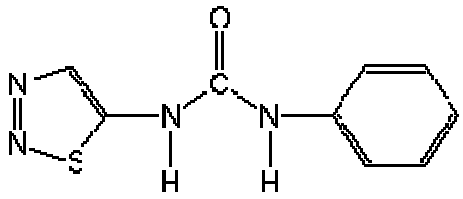
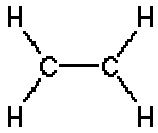
Appendices

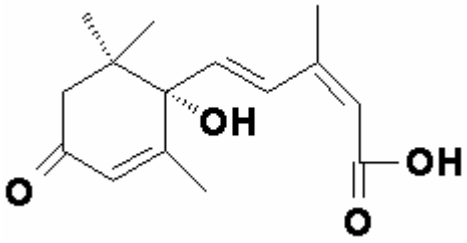
(a) Thidiazuron (TDZ)



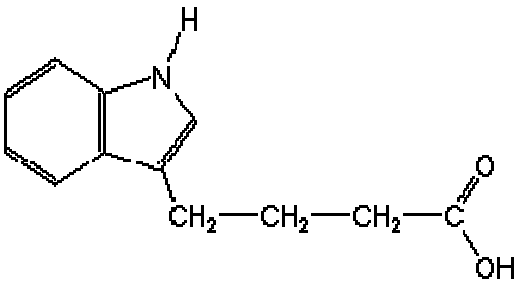
(b) Ethylene (C_2H_4)



(c) Abscisic acid (ABA)



(d) Indole-3-butyric acid (IBA)



Appendix 1. The chemical structures of plant growth regulators (a) Thidiazuron, (b) Ethylene, (c) Abscisic acid and (d) Indole-3-butyric acid.

Appendix 2

Effects of continued exposure to 0.5, 1 and 2 µl l^{-1} ethylene for 4 days, dark storage for 4 days, application of ABA at 25, 50 and 100 µM and TDZ at 5, 10 and 20 µM for 4 days, respectively on leaf chlorophyll content (mg/cm^2) of five *Pelargonium* cultivars.

TREATMENT	'FIRE' Chlorophyll Day 2	'FIRE' Chlorophyll Day 4	'GANYMED' Chlorophyll Day 2	'GANYMED' Chlorophyll Day4	'GRECO' Chlorophyll Day 2	'GRECO' Chlorophyll Day 4	'KATINKA' Chlorophyll Day 2	'KATINKA' Chlorophyll Day4	'SURFING' Chlorophyll Day 2	'SURFING' Chlorophyll Day4
Ethylene										
Control	3.704a	3.499a	3.381a	3.282a	4.861a	4.462a	3.361a	3.193a	3.002a	2.816a
0.5 µl l^{-1} C_2H_4	3.017b	2.856b	2.900b	2.895b	3.915b	3.795b	2.833b	2.778b	2.667b	2.178b
1 µl l^{-1} C_2H_4	2.479c	2.322c	2.539c	2.416c	3.281c	2.847c	2.571c	2.265c	2.369c	1.519c
2 µl l^{-1} C_2H_4	1.875d	1.341d	1.978d	1.483d	2.487d	1.959d	2.056d	1.469d	1.751d	0.921d
Storage										
Control	2.409a		2.203a		2.906a		1.870a		2.467a	
Stored	1.239b		1.009b		1.453b		1.135b		1.507b	
ABA										
Control	3.171a	2.941a	3.101a	2.965a	4.111a	3.951a	2.511a	2.478a	3.301a	3.023a
25 µM ABA	2.616b	2.453b	2.674b	2.359b	3.286b	3.280b	1.951b	1.731b	2.969b	2.682b
50 µM ABA	2.167c	2.062c	2.218c	1.841c	2.871c	2.741c	1.716c	1.163c	2.719c	2.392c
100 µM ABA	1.753d	1.401d	1.789d	1.355d	2.395d	2.051d	1.356d	0.799d	2.246d	1.903d
TDZ										
Control	2.798b	2.165c	3.037b	2.321d	3.398d	3.203d	2.589c	2.166d	3.117c	2.535d
5 µM TDZ	2.896b	3.486b	3.213b	3.815c	3.828c	4.790c	2.768c	3.424c	3.440b	3.956c
10 µM TDZ	3.084b	3.887b	3.304b	4.268b	4.269b	5.241b	3.122b	3.797b	3.469b	4.368b
20 µM TDZ	3.399a	4.491a	3.660a	4.755a	4.979a	5.885a	3.519a	4.328a	3.987a	4.927a

Means separated by LSD (P=0.05). For each cultivar, means followed by with the same letter(s) within days are not significantly different.

Appendix 3

Effects of continued exposure to 0.5, 1 and 2 µl l^{-1} ethylene for 4 days, dark storage for 4 days, application of ABA at 25, 50 and 100 µM and TDZ at 5, 10 and 20 µM for 4 days, respectively on leaf chroma of five *Pelargonium* cultivars.

TREATMENT	'FIRE' Chroma		'GANYMED' Chroma		'GRECO' Chroma		'KATINKA' Chroma		'SURFING' Chroma	
	Day 2	Day 4	Day 2	Day4	Day 2	Day 4	Day 2	Day4	Day 2	Day4
Ethylene										
Control	22.941c	25.015c	27.238b	27.973c	18.316c	20.712c	27.566c	28.438b	30.092b	32.748b
0.5 µl l^{-1} C_2H_4	23.458c	25.098c	28.294b	29.860bc	19.048c	20.834c	29.290bc	29.662b	30.679b	35.850b
1 µl l^{-1} C_2H_4	26.569b	27.604b	29.201ab	30.570b	22.215b	28.109b	29.778ab	31.278b	30.710b	35.897b
2 µl l^{-1} C_2H_4	29.202a	42.925a	31.665a	39.784a	29.409a	37.008a	31.403a	41.965a	36.723a	49.543a
Storage										
Control	27.207b		29.184b		29.034b		30.127b		31.508b	
Stored	42.692a		49.293a		42.143a		42.010a		41.771a	
ABA										
Control	24.409b	25.193c	26.636c	26.652d	21.366a	22.089b	26.611c	29.353d	27.942b	28.066b
25 µM ABA	25.329b	26.313bc	28.101c	29.435c	21.617a	22.174b	29.492b	32.538c	28.428b	28.871b
50 µM ABA	25.837b	27.581b	29.979b	32.705b	22.262a	23.336b	33.384a	41.381b	29.045b	29.211b
100 µM ABA	28.218a	34.960a	32.458a	38.793a	23.553a	30.529a	35.325a	49.098a	31.171a	32.456a
TDZ										
Control	27.137a	30.742a	29.396a	31.674a	24.900a	33.033a	32.051a	46.057a	28.714a	29.172a
5 µM TDZ	25.761b	25.070b	26.989b	24.880b	21.659b	20.209b	27.735b	27.711b	27.899ab	26.544b
10 µM TDZ	25.380b	23.041c	26.921b	24.761b	21.477b	19.480b	27.681b	27.553b	27.846ab	26.308b
20 µM TDZ	24.839b	22.586c	26.333b	24.223b	20.688b	19.147b	27.349b	26.571b	27.510b	25.048c

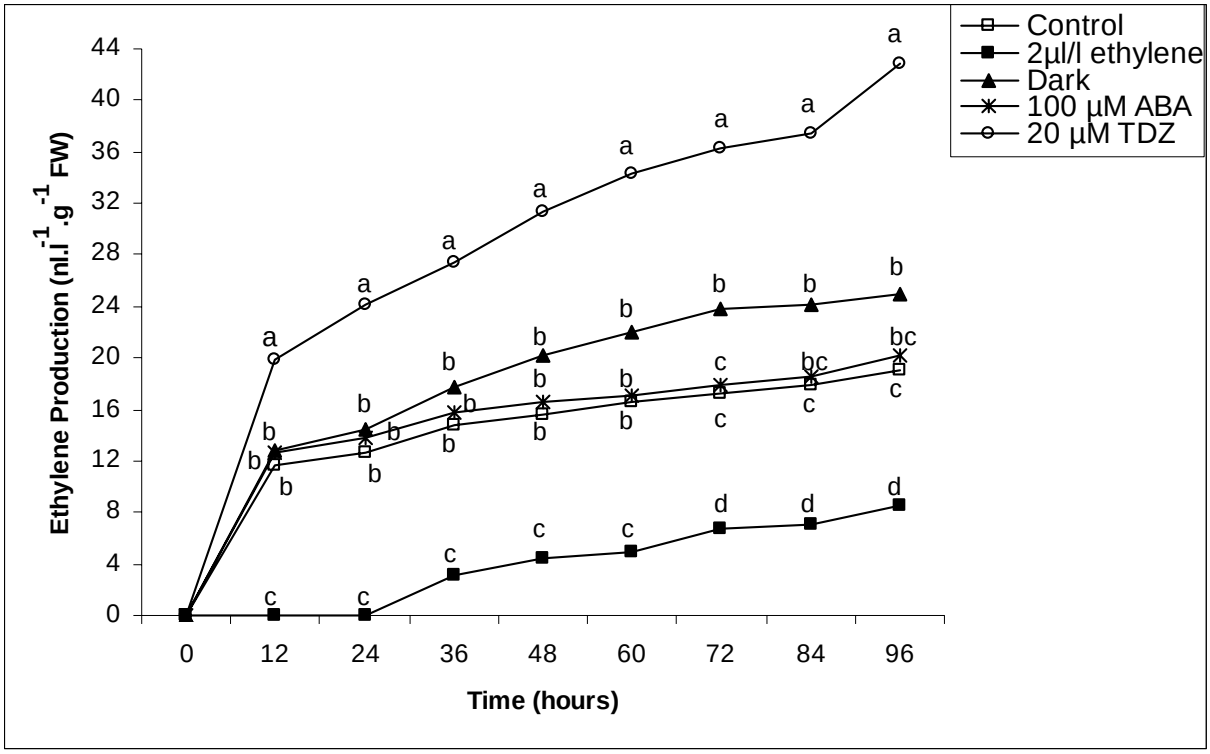
Means separated by LSD (P=0.05). For each cultivar, means followed by the same letter(s) within days are not significantly different.

Appendix 4

Effects of continued exposure to 0.5, 1 and 2 µl l^{-1} ethylene for 4 days, dark storage for 4 days, application of ABA at 25, 50 and 100 µM and TDZ at 5, 10 and 20 µM for 4 days, respectively on leaf hue of five *Pelargonium* cultivars.

TREATMENT	'FIRE' Hue		'GANYMED' Hue		'GRECO' Hue		'KATINKA' Hue		'SURFING' Hue	
	Day 2	Day 4	Day 2	Day4	Day 2	Day 4	Day 2	Day4	Day 2	Day4
Ethylene										
Control	-0.893a	-0.915a	-0.932a	-0.944a	-0.848a	-0.911a	-0.932a	-0.939a	-0.926a	-0.944a
0.5 µl l^{-1} C_2H_4	-0.897a	-0.927ab	-0.934a	-0.948a	-0.873a	-0.911a	-0.941a	-0.942a	-0.941ab	-0.986b
1 µl l^{-1} C_2H_4	-0.927b	-0.941b	-0.947ab	-0.951a	-0.916b	-0.965b	-0.951a	-0.952a	-0.958b	-0.994b
2 µl l^{-1} C_2H_4	-0.947c	-1.088c	-0.957b	-1.029b	-0.967c	-1.078c	-0.954a	-1.062b	-0.994c	-1.175c
Storage										
Control	-0.922a		-0.939a		-0.938a		-0.933a		-0.964a	
Stored	-1.058b		-1.095b		-1.092b		-1.032b		-1.061b	
ABA										
Control	-0.891a	-0.902a	-0.912a	-0.917a	-0.871a	-0.873a	-0.905a	-0.925a	-0.946a	-0.947a
25 µM ABA	-0.893a	-0.909ab	-0.919a	-0.934a	-0.873a	-0.886a	-0.929ab	-0.949a	-0.947a	-0.954a
50 µM ABA	-0.917b	-0.921b	-0.939b	-0.958b	-0.881ab	-0.897a	-0.952bc	-1.027b	-0.953ab	-0.957ab
100 µM ABA	-0.927b	-0.986c	-0.955b	-1.008c	-0.899b	-0.958b	-0.975c	-1.117c	-0.958b	-0.972b
TDZ										
Control	-0.922a	-0.962c	-0.946b	-0.978b	-0.909b	-0.991b	-0.943c	-1.065b	-0.948c	-0.958b
5 µM TDZ	-0.921a	-0.916b	-0.933a	-0.915a	-0.900ab	-0.887a	-0.922b	-0.915a	-0.943bc	-0.935a
10 µM TDZ	-0.918a	-0.909b	-0.930a	-0.915a	-0.893ab	-0.876a	-0.916ab	-0.911a	-0.939ab	-0.932a
20 µM TDZ	-0.917a	-0.893a	-0.928a	-0.909a	-0.888a	-0.869a	-0.905a	-0.900a	-0.934a	-0.929a

Means separated by LSD (P=0.05). For each cultivar, means followed by the same letter(s) within days are not significantly different.



Appendix 5. Ethylene production in *Pelargonium zonale* 'Katinka' cuttings that were untreated (control) or treated with 2 µl l^{-1} ethylene, dark storage, 100 µM ABA and 20 µM TDZ for 4 days. Means separated by LSD (P=0.05). For each cultivar, means followed by the same letter(s) within days are not significantly different.

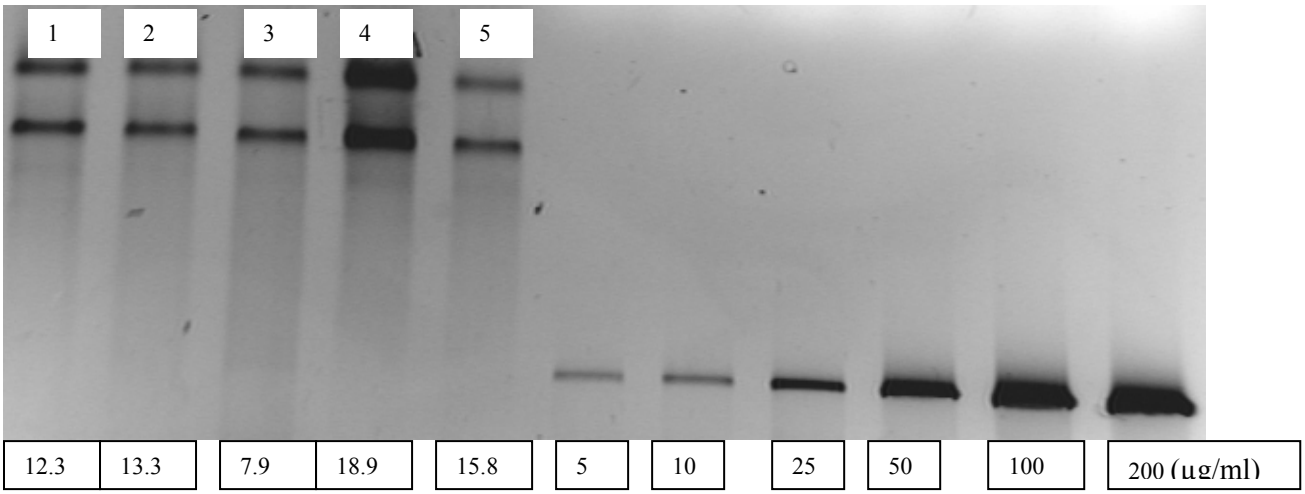
Appendix 6.

Total RNA concentration from the leaves of *Pelargonium zonale* cvs 'Katinka' and 'Ganymed' after exposing them to various treatments.

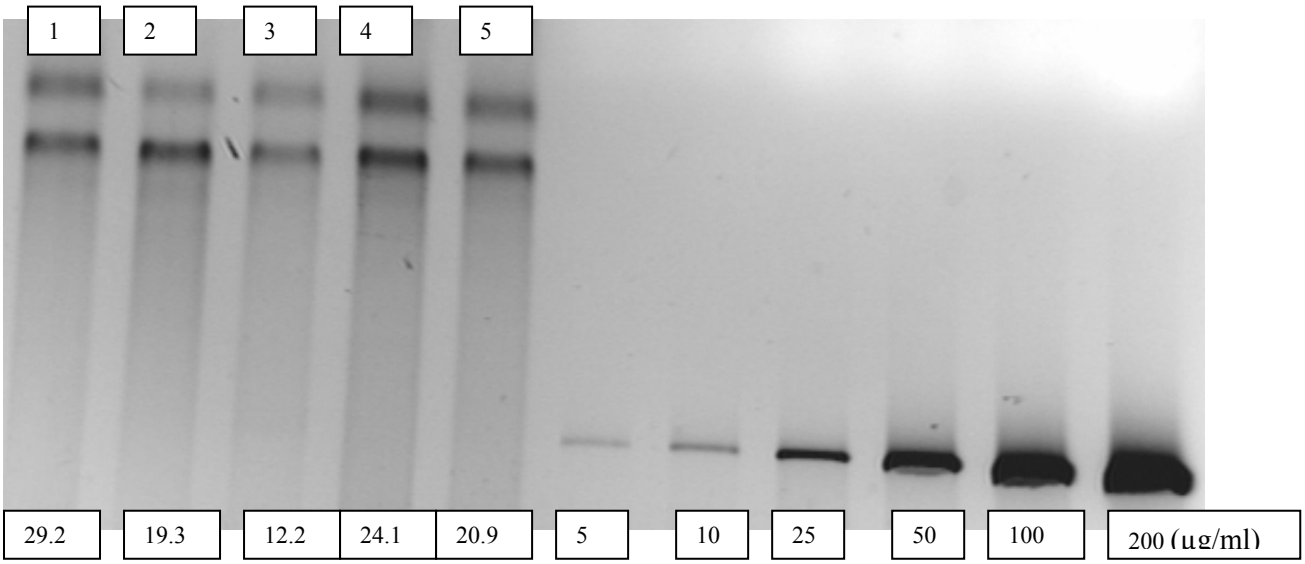
SAMPLE NO.	CULTIVAR	TREATMENT	CONCENTRATION (µg/ml)	CONCENTRATION X 10 Dilution Factor (ng/µl)
1	'Katinka'	Control	12.2703	122.703
2	'Katinka'	2 µl/l C_2H_4	13.2723	132.723
3	'Katinka'	Dark Storage	7.9387	79.387
4	'Katinka'	100 µM ABA	18.8962	188.962
5	'Katinka'	20 µM TDZ	15.8419	158.419
6	'Ganymed'	Control	29.2207	**29.2207 (no dilution)**
7	'Ganymed'	2 µl/l C_2H_4	19.33	193.3
8	'Ganymed'	Dark Storage	12.1745	121.745
9	'Ganymed'	100 µM ABA	24.1046	**24.1046 (no dilution)**
10	'Ganymed'	20 µM TDZ	20.9152	**20.9152 (no dilution)**

Measurements were done using a SmartSpec™ 3000 Spectrophotometer (Biorad, California).

(a). ‘Katinka’ leaves.



(b). ‘Ganymed’ leaves.



Appendix 7. Total RNA concentration by comparing with standard concentrations of λDNA from the leaves of *Pelargonium zonale* (a) ‘Katinka’ and (b) ‘Ganymed’ after exposing them to various treatments. Quantification of RNA and detection of presence of DNA in lanes (1) control, (2) 2 µl l^{-1} ethylene, (3) dark storage, (4) 100 µM ABA and (5) 20 µM TDZ was done by loading total RNA into a 1% agarose gel and performing flatbed electrophoresis using 1XTAE buffer.

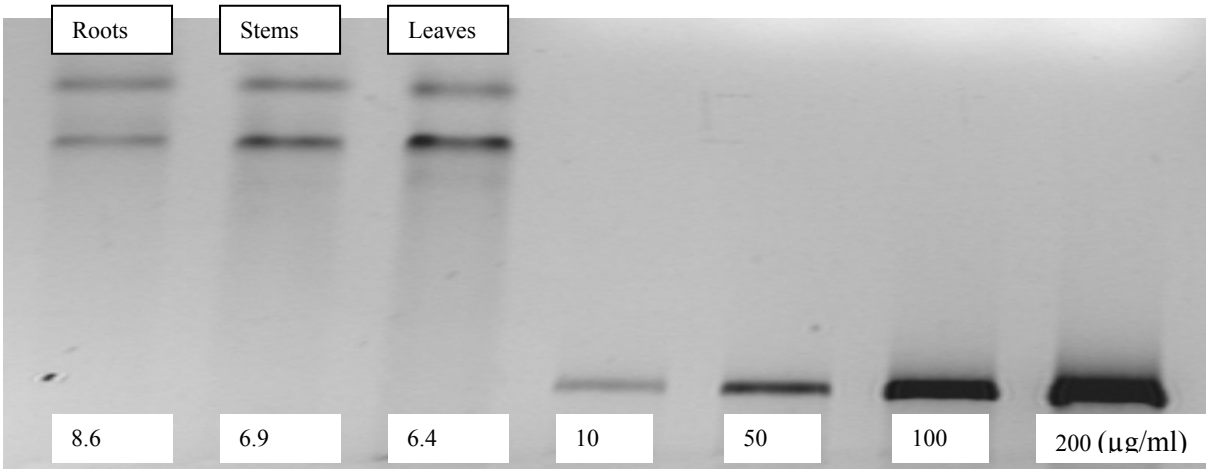
Appendix 8.

Total RNA concentration from various organs of *Pelargonium zonale* 'Katinka'.

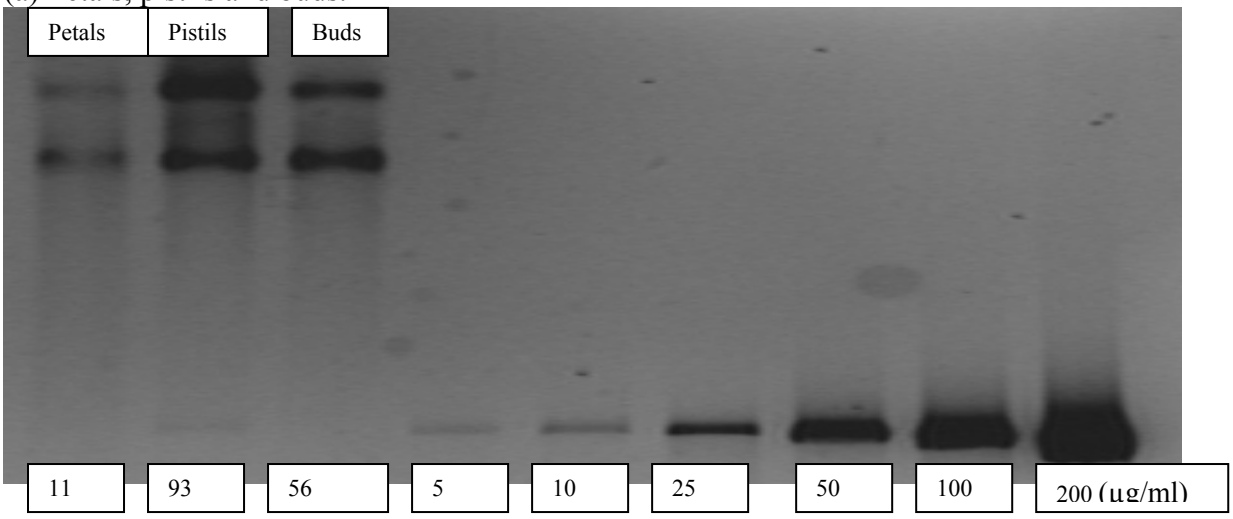
SAMPLE NO.	ORGAN	CONCENTRATION. (µg/ml)	CONCENTRATION. X 10 Dilution Factor (ng/µl)
1	Roots	8.569	85.69
2	Stems	6.989	69.89
3	Leaves	6.407	64.07
4	Buds	56.0398	**56.0398 (no dilution)**
5	Petals	11.6996	**11.6996 (no dilution)**
6	Pistils	93.2166	**93.2166 (no dilution)**

Measurements were done using a SmartSpec^{TM} 3000 Spectrophotometer (Biorad, California).

(a) Roots, stems and leaves.



(a) Petals, pistils and buds.



Appendix 9. Total RNA concentration by comparing with standard concentrations of λDNA from various organs of *Pelargonium zonale* 'Katinka'. Quantification of RNA and detection of presence of DNA in (a) roots, stems or leaves, (b) petals, pistils or buds was done by loading total RNA into a 1% agarose gel and performing flatbed electrophoresis using 1XTAE buffer.

Appendix 10.

Three fragments obtained after amplification of degenerate primer pair ACSd2 using genomic DNA of *Pelargonium zonale* 'Katinka'.

GENE NAME	GENOMIC DNA (bp)	mRNA (bp)	REMARKS
PzACS3	590	374	New partial putative ACC gene
PzACS4	745	374	New partial putative ACC gene
PACSD2_8	803	374	Entirely homologous to pGAC-2G (Accession No. U17230)

(a). PACSD2_8 genomic DNA

```
TTCCAAGATTATCACGGGTTGCCAGAGTTCAGATATGTAAGTACATATATAAAATACATACATCAAATCTGTGTACAAAAATT
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TGTCACCGTCCCAATCACCTGTTGGATCCTACTAGTATCATTATTGGTGTTGCCATGATACCAAAAATTAGGATTTGCTAAAA
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AGCTAATTCCTGTAGTCTGTGAAAGTGAGAACAATTTCAGGATCACCCGAAGTGCCTTAGAAGAAGCCTATGAGAGAGCTCAA
GAGGACAACATTAGAGTCAAGGGATTGCTCATAACAAACCCCTCCAATCCCCTCGGCACA
```

(b). PACSD2_8 mRNA

```
TTCCAAGATTATCACGGGTTGCCAGAGTTCAGATATGTAAGTACATATATAAAATACATACATCAAATCTGAAACAGAGTAAC
ATTTAACCCAGATCGCATAGTTATGAGTGGAGGAGCAACTGGAGCTCATGAAATGATTGCCTTCTGTTTGGCTGATCCTGGCG
ATGCTTTTCTTGTCCCAACTCCTTATTATCCTGGATTTGATAGAGACCTGAGGTGGAGAACTGGTGTGCAGCTAATTCCTGTA
GTCTGTGAAAGTGAGAACAATTTCAGGATCACCCGAAGTGCCTTAGAAGAAGCCTATGAGAGAGCTCAAGAGGACAACATTAG
AGTCAAGGGATTGCTCATAACAAACCCCTCCAATCCCCTCGGCACA
```

(c).PzACS2 Amino acids (126)

```
FQDYHGLPEFRYVSTYIKYIHQIZNRVTFNPDRIVMSGGATGAHEMIAFCLADPGDAFLVPTPYYPGFDRDLRWRTGVQLIPV
VCESENNFRITRSALEEAYERAQEDNIRVKGLLITNPSNPLGT
```

(d) PzACS2 amino acid sequence compared to those of CsACS1

```
PzACS2          FQDYHGLPEFRYVSTYIKYIHQIZ-NRVTFNPDRIVMSGGATGAHEMIAFCLADPGDAFL 59
CsACS1_         FQDYHGLPAFK--KALVEFMAEIRGNKVTFEANNIVLTAGATSANETLMFCLAEAGDAFL 58
                ******** *:  .: :::: :*  *:***:.:.**::.***.*:* : ****:.*****

PzACS2          VPTPYYPGFDRDLRWRTGVQLIPVVCESENNFRITRSALEEAYERAQEDNIRVKGLLITN 119
CsACS1_         LPTPYYPGFDRDLKWRTGVEIVPIHCTSSNGFQVTQPALEQAYQEAQARNLRVKGVLVTN 118
                :************:*****:::*: * *.*.*::*:.***:**:.**  *:****:*:**

PzACS2          PSNPLGT 126
CsACS1_         PSNPLGT 125
                *******
```

Appendix 11. Sequence analysis of *Pelargonium zonale* 'Katinka' 803 bp genomic DNA fragment. (a) nucleotide sequence, (b) mRNA sequence, (c) deduced amino acid sequence (d) homology comparison with *CsACS1* (AB032937*)* sequence using CLUSTAL W programme. *= identical amino acid. Border between intron and exon sequence was detected with splicing specific sequence GT and AG, respectively. : = two nucleotides out of the triplicate amino acid code are identical. . = one nucleotide out of the triplicate amino acid code are identical.

(a). β-Actin (PoAc97, X55751), DNA

GTCGACATACTATTATTCAATTTATCTGCGGCCTTTACTTTGTCATCTGTGCAAAGAGAGAATATATTTCCCTGA
AAACTGAAAAAATAGAGGTTCGAAGTAATAGTAACCTTTGACTGAAGGAATAGTATATGAACCTCTCTTTCCCAT
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TCTTCTGTTGAGAAGAGCTATGAGCTCCCAGATGGGCAGGTGATCACCATTGGTGCTGAGCGTTTCCGGTGTCCT
GAGGTCCTTTTCCAACCTTCAATGATTGGAATGGAAGCTGCAGGAATCCACGAGACTACATACAACTCTATCATG
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ATTGCTGATAGAATGAGCAAAGAAATTACTGCATTGGCTCCTAGCAGCATGAAGATTAAGGTGGTCGCTCCACCA
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CCTTCTATTGTCCACAGGAAGTGCTTCTAATTTTTCCAAGATTGACAATGTTGGTGAAAGGAAAATACTTCTTAT
TTCCTACTGGATCAGAAATGCAATTGCAGTGTTATATTCTAGCTTTATTTTCTGTATTTTTGTTCTCATGCTGGA
TTGAAGATATTGAGAGGGCAGAGTTGATTGTTGGGTTATGTTAATTCTTTTATTTCTATTGACTTTTTCTACTCT
TCCGTTATTCTTCCTTTTTCTGTGGCCCCTCCCAACCCCATTTGAGGTAACTGAAGATTGGTATAAAAACATATT
CGCCTCACGGTAAATTTATGTATTTTTTCACATCATTAAATCATTTCACTATGGTAAGTTAAGTTTGTTCGGTGT
AGACACTTGTATTTACTATGAAAATGCTTTAACTTAGGCAAGAATCTTATTTATGGTTGATTAGTGATTAAGGCA
TGTTAATTGTGATGGGTACCATGATTAATCATAGCAATTATTTAAACCTTGTTGAATCAAAGTAGCTTGGCCGAA
GATCCTAGAACTTAAATAAAAGGAAAGAATTC

(b). *PzACS1* (pGAC-1, U17299), mRNA

TATCACTACTCTCGCTTCTGAGTGCCTAATTATTTTTGTCCAAGCTCTCAGTACGTACGTGTTGTACGTGTTTAC
ATAGATGGAGAACAAGAGCAAACAGCTTCTGTCAAAGATTGCAACCAACGACGGACACGGCGAGAACTCCCCATA
TTTCGATGGTTGGAAGGCTTATGACCGTGATCCGTTCCATCCGTCTCAGAATCCTAACGGTGTTATCCAGATGGG
TTTAGCTGAAAATCAGCTTTCATCTGACTTGATTGAAGATTGGGTGAGGTCCAACCCAGAAGCCTCAATCTGCAC
TCTTGAGGGAGTTGGTAAGTTCAAGGACGTAGCTAACTTTCAGGACTACCATGGCCTGCTGGAGTTCAGGCACGC
CGTGGCTAAATTTATGAGCAGAGGAAGGGGCGGGAAGGTCACATTTGATCCCGACCGTGTCGTCATGAGCGGCGG
AGCCACCGGAGCCAACGAGCTCATCGTCTTCTGTTTGGCCAATCCCGGCGACGCTTTCCTTCTCCCATCTCCTTA
TTATCCAGCAAACGACCGTGACTTGCAGTGGCGAACCGGAGCTCAGATCATTCCGGTGCACTGCAACAGCTCCAA
CGGTTTCAAGATAACCAGAGAGGCACTAGAAAGATCATACGCACAAGCACAAGAAAGCAACATAAACGTAAAAGG
CGTGCTCTTAACCAACCCATCGAACCCTCTAGGCACAATTCTGGACCGCGACACTCTCAAGAGCATCGTCAGCTT
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GTCCAAGGACTTGGGCATGCCCGGGTTCCGCGTCGGGATCGTGTACTCATTCAACGACGACGTCGTATCCTGCGC
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TTTCGTGGAGCGGTTCCTCGCGGAGCGGAGGCGGTTGGGGAGGAGGCACGGCGTGTTCACGAAAGGGCTCGAGGA
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GACGTTTGAGGCGGAGATGGTGCTGTGGAAGGTGATTATTAATGAGGTGAAGCTAAACGTGTCTCCGGGGTCGTC
GTTTCATTGCGTGGAGCCGGGTTGGTTTAGGGTTTGCTTTGCCAACATGGACGACGAGACGGTCCACGTGGCGCT
GAAGAGGATCAGGGCGTTTGTGAGGAAGAAGGAGGTGGGTCCGGTGAAGAGGAAGAGGTTCATGGACAACCTTAA
CCTCAGGCTGAGCTTCTCGTCGCTAAGGTACGATGAGAGTGTGATGTTGTCGCCGCACATAATGGGTCCACGCAC
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GGAATTGGTACATATTTTAGGGTTTGCTGATGTTTTCTTTCATCATGAATTGGTACATATTTATGATGTTCAAGG
CTCCAAGTGATGGATACATGGAGGATTCATTTGGATGCATGCCTTGCAAGAGTCAGCAATCTTTGTTAATTAGTG
TATGGTTTGTGATAATAAAGATGCAAAATTCTGTGTTGTTTTA

(c). *PzACS2* (GACS2, U88971), mRNA

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TGGACATGGCGAAAACTCACCTTACTTTGATGGCTGGAAAGCTTACGACAACAATCCTTTCCATCTCACCCAAAA
CCCTCAAGGTGTCATCCAGATGGGCCTCGCAGAAAATCAGCTTTCTTTCGAGTTGATTGAGCAATGGGTCCTTAA
CAACCCACAAGCCTCCATTTGCACAGCACAAGGTCTGCAAGAATTCAAGGACACTGCAATCTTTCAAGATTACCA
TGGCTTGCCAGAGTTCAGATATGCTGTTGCAAATTTCATGGGAAAGGTGAGAGGAAACAGAGTAACATTTAACCC
AGATCGCATAGTTATGAGTGGAGGAGCAACTGGAGCTCATGAAATGATTGCCTTCTGTTTGGCTGATCCTGGCGA
TGCTTTTCTTGTCCCAACTCCTTATTATCCTGGATTTGATAGAGACCTGAGGTGGAGAACTGGTGTGCAGCTAAT
TCCTGTAGTCTGTGAAAGTGAAAACAATTTCAGGATCACCCGAAGTGCCTTAGAAGAAGCCTATGAGAGAGCTCA
AGAGGACAACATTAGAGTCAAGGGATTGCTCATAACAAACCCATCAAACCCACTAGGAACTATCCTGGACAGAGA
GACACTAGTCAGTCTAGTGAGCTTCATCAATGAAAAGAACATTCACTTGGTCTGTGATGAAATCTACGCCGCCAC
AGTCTTCTCTCAGCCCGCTTTCGTTAGCATTGCTGAGGTTATCGAGCAAGAGAACGTTTCGTGCAACCGCGACCT
CATCCACATTGTCTACAGCCTGTCCAAGGACATGGGCTTCCCTGGCTTCAGGGTGGGGATTGTCTACTCCTACAA
TGACGCAGTTGTGAATTGTGCGCGAAAGATGTCAAGTTTCGGCCTTGTATCCACACAAACTCAGCACCTAATCGC
ATCAATGCTCTCGGACGATGAATTCGTGGACACATTCATCGTGGAGAGCGCGAAGAGGCTAGCGAGAAGGTACAC
AACCTTCACAAGAGGGCTTGCACAAGTGAACATTGGATGCCTAAAGAGCAATGGGGGGTTATTCATATGGATGGA
CTTGAGGAGGCTTCTCAAGGAGAAGACTTTCGAGGCGGAGATGGCTCTGTGGAGAGTGATAATCAATGAAGTGAA
GCTAAATGTGTCGCCAGGGGCGTCGTTCCATTGCTCGGAGCCAGGGTGGTTTAGAGTGTGCTTTGCCAACATGGA
TGACTTGACGATGCAGGTGGCTCTGAGGAGGATCATAACATTTGCACTTCAGAACAAGGAAGCTGCGGTTTTGCC
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GATGTCTCCAATGTCCCCTCACTCCCCAATACAATCACCACTTGTGAGAGCCACTTAGAAACACATGAATAATAG
AGAATAACGGGCGATGCGGCCGCCAAAAATAGGTTGATCTATGTATGCATTAACGTTTTTAGTTAATCTGTGTTT
ATATGTATAACAAGAAGGAACAAAATGTATTCTTTCTGTATAAATAACCCAAACTTAGAAGATGCTTGCTGTGCA
TCCTTCTGGGAAAAAAAAAAAAAAAAAAAAA

(d). PzACS3 (new), DNA, Partial cds

GTTCCAAGATTACCACGGGTTGCCTGCATTCAAACAAGTAAGCACTGACACATAAAACGTGTGCACTTTGACTGA
TTTAAAAAAAAAAATGTACATTTTCTAACGATATTGTACTTTTCAGGCATTGGTCGATTTTATGTCCCAAATAAGA
GGAAACAAAGTGACATTCGACCCGAGCAAGCTAGTCCTCACCGCGGGTGCCACCTCAGCCAACGAGGCTCTCATG
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AGCCCGAAAACAGAACCTGAAAGTGAAGGGCGTGCTCGTGACCAACCCCTCCAATCCCCTCGGCAC

(e). PzACS4 (new), DNA, Partial cds

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ATATATTTCCACAATAATTTGAAAACGCGTCTACACGTTATATATATGGGATATATAATGAGTGGATGTTGCGTA
GTTGTTTATATAATTGACATCAAACTGAGGTAGCTAGGTTGGAATGTACTTATGCAGGAAATGGTGGAGTTCTTG
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GAGTCCCTCGTGTTTTGTCTAGCTCAACCTGGTGATGCTTTCCTTCTTCCCACTCCTTACTATCCCGGGTAATCA
TTTTACATATGGTCACAATTTAAAGTAGCCTTTTGATTTTAAGTTTTGGTTTTATGTGAATCACATTCAACAATG
TTATTTCCGTTATTTTTTCACATAAAACATTTTCATAATCACACTCTATTTACTGTTTTTAAGTAGTCCACATCT
CAATATACCTTTCATTCCTCACGTGTTTATTTGTGTGTGAGACAGGTTTGATAGAGATCTCAAATGGCGAACCGG
AGCTGAAATCGTTCCCATCCATTGTTCGAGCTCTAACAATTTCCGAATCACTCCTTGTGCGCTACAAGAAGCTTA
CGAGCGAGCTCAAAAACTAGGCCTAAACCCGAAAGCAGTACTGATCACCAACCCCTCCAACCCCCTCGGCAC

(f). *PzETR1* (PhETR1, AF141928), mRNA

CTCTCTTAATCGAATCGAAGGGAAGAAGAAGAATCAGATTGATCTTCCGGAGGTTTTGGTGTGAGCCTCGCGTGG
CTCACTCAGTCAAATAGCCATAGATGATCGCTGAATTGCTGAGAATTCTCGTCCCTTGATTGAAAATTAAGTAAT
GCTTAAGAGGAAAAATTGACTGATTCTGTTCAATTCAACGGCAATGGAGGCTTGCAATTGTTTTGAGCCACAATG
GCCGGCTGATGACCTTTTAATGAAATACCAATATATATCAGATTTTTTTATCGCGGTTGCTTACTTCTCAATCCC
TTTAGAGCTGATCTACTTCGTTAAAAAATCGGCAGTGTTTCCATATAAGTGGGTCCTTGTGCAGTTTGGTGCTTT
CATAGTTTTGTGTGGGGCAACACATCTTATTAACTTATGGACTTTTAACTTGCATACAAGAACTGTGGAAATAGT
AATGACCACCGCAAAGCTTATGACTGCTGCAGTCTCATGTGTGACTGCCCTTATGCTAGTTCACATTATTCCTGA
TCTGTTGAGTGTTAAGACTAGAGAACTATTTTTGAAAAACAAGGCTGCAGAGCTTGATAGAGAAATGGGTCTGAT
TCGCACACAGGAAGAGACTGGTCGACATGTTAGGATGCTGACTCATGAAATCAGAAGCACACTTGATAGACATAC
TATACTAAAGACCACTCTTGTTGAGCTGGGTAGAACTTTGGGATTGGAAGAATGCGCCTTGTGGATGCCAACACG
GACTGGTTTAGAGCTTCAACTTTCACATACTCTTCGTCAACAGAATCCAGTGGGATATACAGTACCCATCCATCT
TCCTGTGCTTAATCAGGTTTTTAGTAGCAACCGGGCAATAAAAATATCACCAAATTCCCCTATAGCAAGGTTACG
ACCTCTTGCAGGAAAATACGTACCTGGAGAAGTTGTTGCTGTTCGGGTCCCGCTTTTACATCTTTCCAATTTCCA
AATCAATGACTGGCCTGAACTTTCAACAAAACGTTATGCTATGATGGTTTTGATGCTTCCATCGGATAGTGCAAG
GCAATGGCATGTCCACGAATTGGAGCTGGTGGAAGTAGTAGCTGATCAGGTAGCAGTAGCACTTTCACATGCTGC
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AGCAGAAACAGCAATTCGTGCTCGCAATGACTTCTTGGCTGTTATGAACCATGAGATGAGAACTCCTATGCATGC
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TATAGAGAGATAAATACATTGTCAAGCTTTTGGAGATCTTAAAAACCAATAATAGGTCTTTTATGCATAAATTTT
GCTTGTTTTGGGTTTTAAAAA

(g).*PzETR2* (PhETR2, AF141929), mRNA

AAAAAGTAGCAGATCTTCATAAGGATTGGGTGTGGGTCTCTCGTGGCTCTCTGACACAGAAAGCCATAGCTGATA
GCTCCTAATCTCTCAAGCTCTTATTCTCTCTCTACATCGCTTCTGCAGAAGCAAGGTTTGCATAAGAGGAAGATT
TGACTAATTTGATTCAATTATATTGGGATGGAGTCTTGCAATTGCATTGAGCCACAATGGCCTGCCGATGAGTTA
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TTCGTAAAGAAATCTGCTGTGTTTCCCTACCGATGGGTCCTAGTTCAGTTTGGTGCTTTCATAGTTTTGTGCGGG
GCTACACATCTTATTAACTTGTGGACTTTTAACATGCATTCAAAAACTGTGGAAATAGTAATGACCACCGCAAAG
ATTATGACAGCTGTCGTGTCATGTGCTACCGCTCTTATGCTGGTTCACATAATTCCTGATCTGTTGAGTGTTAAG
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ACTGGTCGACATGTAAGGATGCTGACTCATGAGATCAGAAGCACTCTCGATAGACACACTATATTGAAGACCACT
CTTGTTGAACTGGGTAGAACTTTGGCATTGGAAGAATGTGCCTTGTGGATGCCAACGCGTACCGGTTTAGAGCTT
CAACTTTCATATACTCTTCGACAACAGAATCCAGTTGGATTTACTGTACCCATCCATCTTCCCGTGATTAATCAA
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TTGGCTACTCTTATAAATGACGTGTTGGATCTCTCCAGGCTAGAAGATGGGAGCCTTCAACTTGACATTGGAACT
TTTAATCTCCATGCTTTACTCCGAGAGGTTCATAACTTAATCAAGCCTATTGCATCTGTCAAGAAGCTGTGCATA
TCATTGAATGTAGCTACCGATCTGCCCGAGTATGCCGTTGGTGATGAGAAGCGGCTGGTGCAGATAATTCTGAAT

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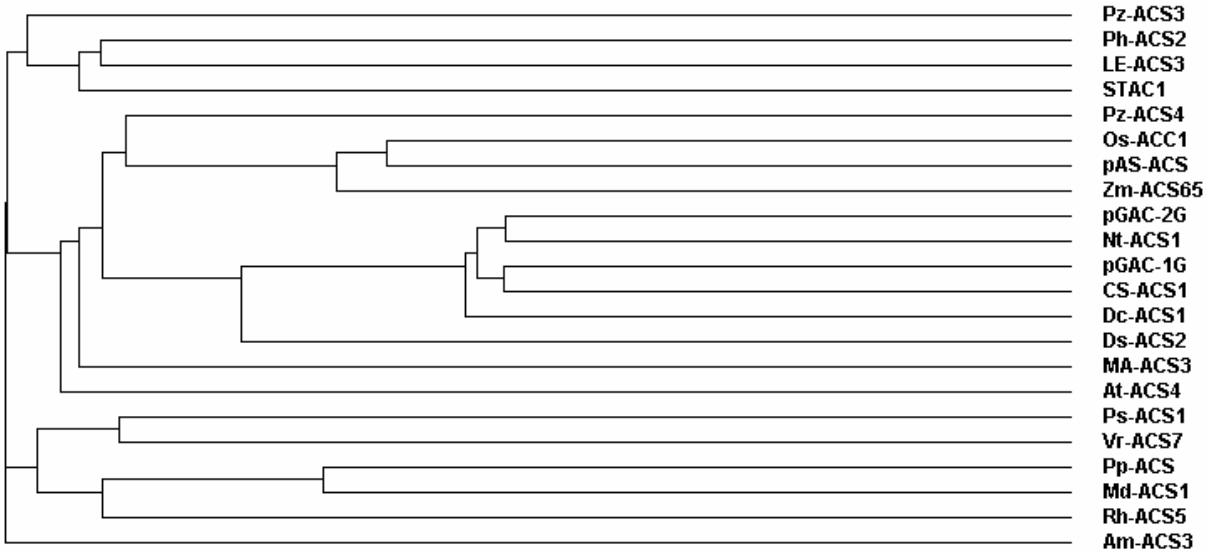
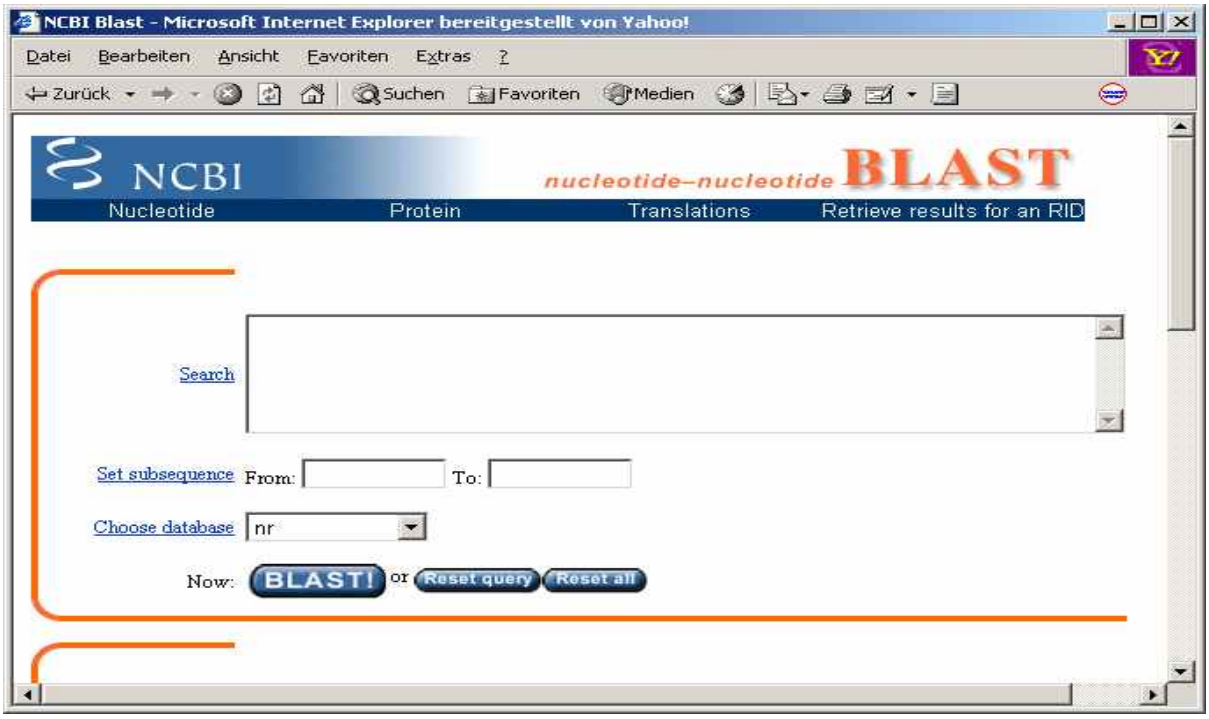
TGGATTGATAGTGAAGGCCCCGGCAAAGGATGCACTGCTACTTTTGTAGTAAAACTCGGGATTCCAGAACGATCT
AGCGAACCTAAGCTGCTGTTGATGCCTAAAGTGCCAGCAAATCACGGGCAGACAAATTTTTCAGGGCTCAAAGTT
CTTTTGCTGGATGATAACGGCGTAAGCAGGGCAGTGACAAGAGGACTTCTTGCGCACCTGGGATGTGATGTGACG
ACTGTAAGCTCCAGTGACGAGTTGTTGCGGGTCGTCTCTCAGGACTACAAGGTGGTCTTCATGGATGTCTGCATG
CCCGAAGTGGACGGTTTTGAAATTGCTGTCCGCATCCATGAAAAGTTCATGACGCGCCACGAGAGACCCCTCATA
GTGGCATTGACTGGCAACATAGACCAGGTAACCAAGGACAACTGCACGAGAGTTGGAATGGAAGGCGTTGTGTTG
AAACCTGTTTCAATCGATAAAATGCGGAATGTTTTGTCTAACCTTTTAGAGCACCGTGTGCTATTTGAGGCCATT
TAGGCGGATTTGAAGCTCCTGATCATTTTGTGCATACCATAACTGCATTGAAGAAAATGGAGCATTGGTGAAAAT
AACGGACCATACCGAAAGCATCTAAGCAAAGGTGATGCCTTTCTAGTTAGGACGGGAGAGAGAGAAAATGGCGGG
GGTATTTTGCCATAATACTGCTTTAGGAGACAAGTTGGGTGTTATTGCTTGGCTGTAAAAAACTGATTGGTCTTT
TTATATACAATGGATTTTTCTCAAAAAAAAA

Appendix 12. Gene sequences obtained from gene bank and used for the construction of gene specific primer pairs (a) β-Actin, (b) *PzACS1*, (c) *PzACS2*, (d) *PzACS3,* (e) *PzACS4,* (f) *PhETR1* and (g) *PzETR2*.

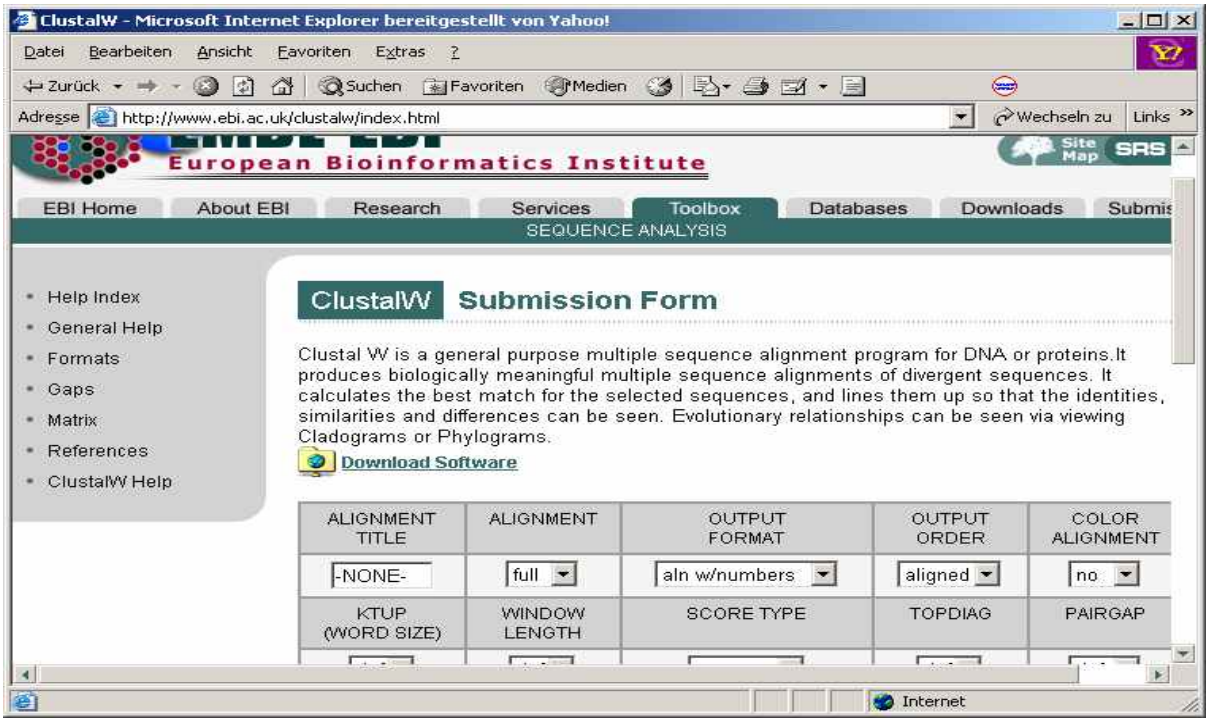


Appendix 13. Dendrogram of various amino acids sequences of ACC synthase genes from different plant species. Gene bank names and their Accession numbers: *Pz-ACS3* Pelargonium (new); *STAC1* Potato (Z27233); *Pz-ACS4* Pelargonium (new); *LE-ACS3* Tomato (L34171); *Ph-ACS2* Petunia (AF049711); *Vr-ACS7* mung Bean (AF 151961); *Rh-ACS5* Rose (AY525069); *pGAC-2G* Geranium (U17230); *pGAC-1G* Geranium (U17228); *Cs-ACS1* Cucumber (AB032937); *Nt-ACS1* Tobacco (X65982); *Dc-ACS1* Carnation (Z18952); *Ds-ACS2* Orchid (L07883); *Os-ACC1* Rice (M96673); *Zm-ACS65* Maize (AY359571); *pAS-ACS* Asparagus (AB111528); *MA-ACS3* Banana (AB021908); *Ps-ACS1* Pea (AF016458); *Am-ACS3* Snapdragon (AF083816); *At-ACS4* Arabidopsis (U23482); *Pp-ACS* Pear (AB015624); *Md-ACS1* Apple (U89156).

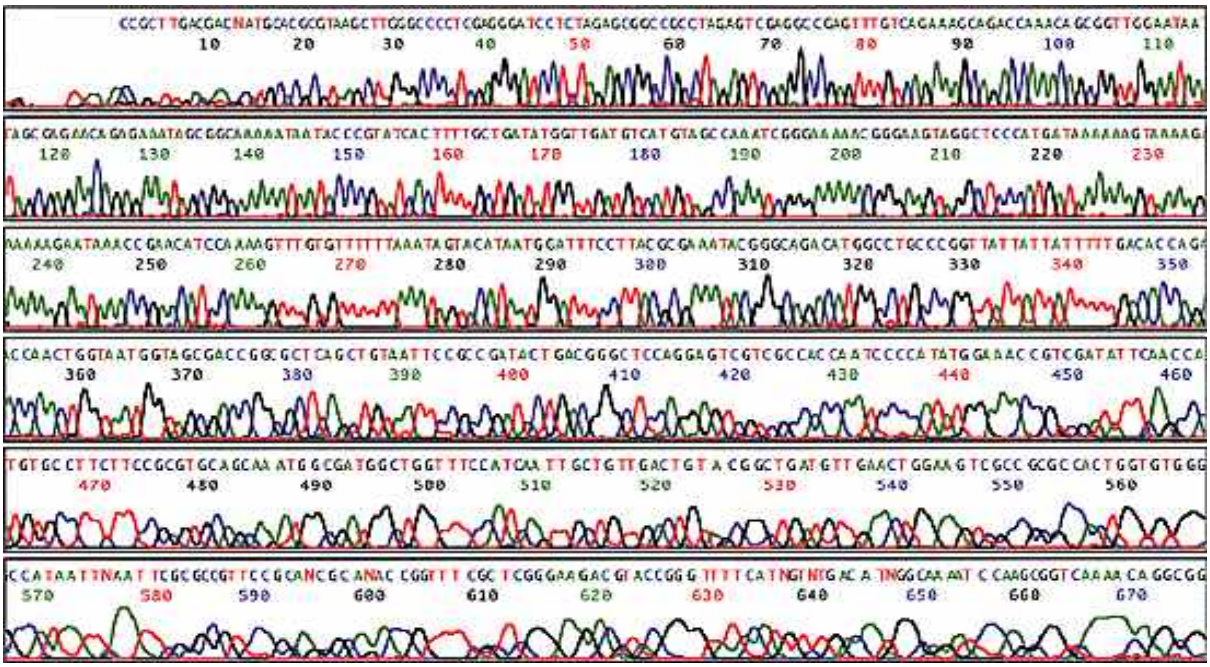
(a). NCBI Blast program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?CMD=Web&LAYOUT=TwoWindows&AUTO_FORMAT=Semiauto&ALIGNMENTS=50&ALIGNMENT_VIEW=Pairwise&CLIENT=web&DATABASE=nr&DESCRIPTIONS=100&ENTREZ_QUERY=%28none%29&EXPECT=10&FILTER=L&FORMAT_OBJECT=Alignment&FORMAT_TYPE=HTML&NCBI_G).



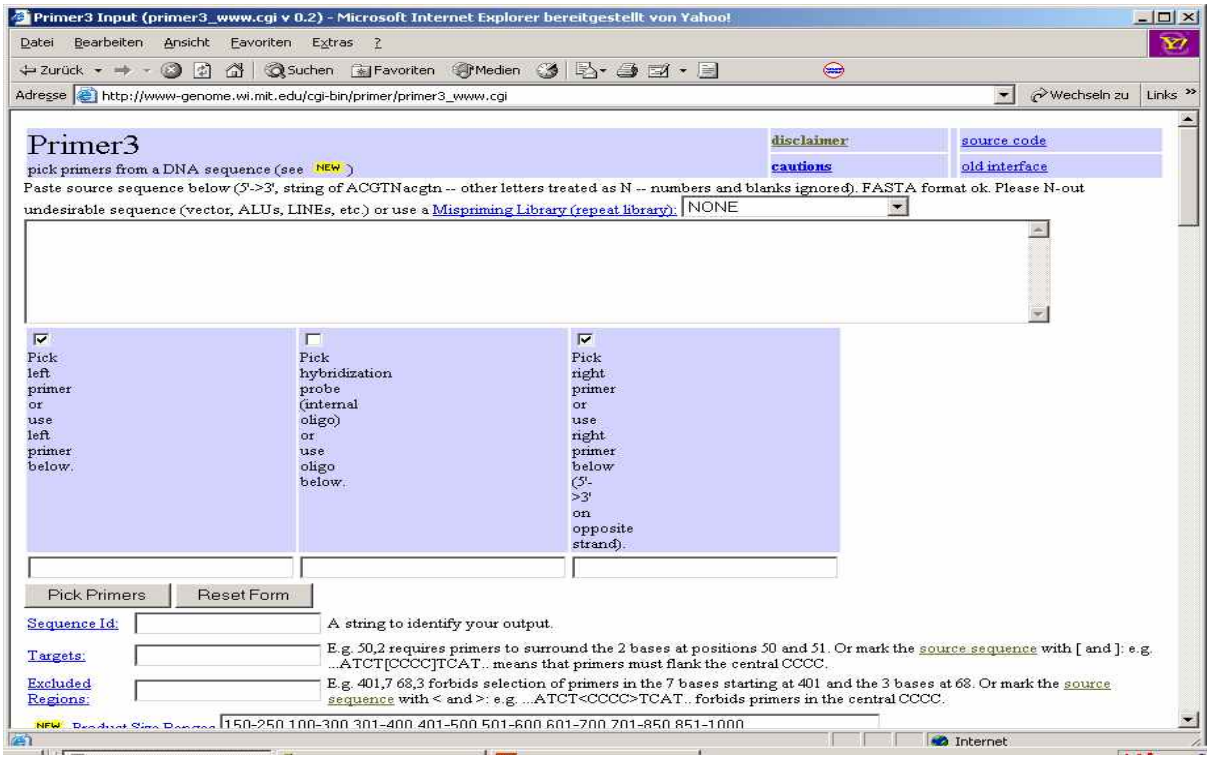
(b). ClustalW program (http://www.ebi.ac.uk/clustalw/).



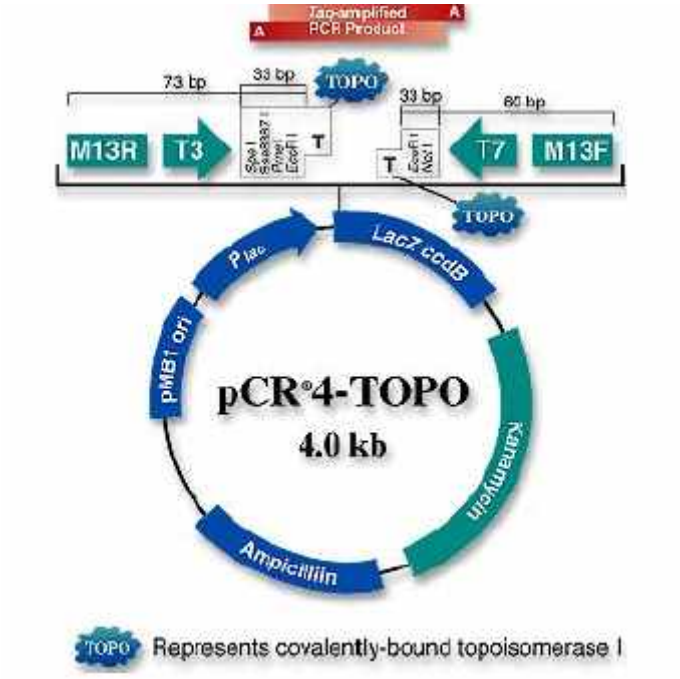
(c). Chromas (http://www.technelysium.com.au/chromas.html)



(d). Primer 3 Input program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).



Appendix 14. Computer programs used for analysing gene sequences (a) NCBI Blast program, (b) ClustalW program, (c) Chromas and construction of primers (d) Primer 3 Input program.



Appendix 15. The principle of ligation into a Topo TA Cloning Kit (Invitrogen, Carlsbad, CA, http://www.invitrogen.com/content/sfs/vectors/pcr4topo_map.pdf).

Publications

Mutui, T. M., Mibus, H. and Serek, M. 2005. Isolation, characterization and expression studies on ACC synthase and Ethylene Receptor (ETR) genes in *Pelargonium*. Molecular Breeding (manuscript in preparation).

Mutui, T.M., Mibus, H. and Serek, M. 2005. Effects of thidiazuron, ethylene, abscisic acid and dark storage on leaf yellowing and rooting of *Pelargonium* cuttings. *Journal of Horticultural Science & Biotechnology* (accepted).

Mutui, T.M., Mibus, H. and Serek, M. 2005. Effects of thidiazuron, ethylene, abscisic acid and dark storage on leaf yellowing and rooting of Pelargonium cuttings. 42 Deutsche Gartenbauwissenschaftlice Tagung (42 *German Society for Horticultural Science*), Geisenheim, Germany, 23.02.2005 -26.02.2005. BHGL – Schriftenreihe Band (*Book of Abstracts*) 23, pp. 139. ISSN: 1613-088X.

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides Statt, dass ich die vorliegende Arbeit selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe sowie dass diese Arbeit noch nicht als Dissertation oder andere Prüfungsarbeit vorgelegt worden ist.

Hannover, den 26. 09.2005

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Lebenslauf

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1995 - 1998:	DAAD-Stipendium zum Magisterstudium, Universität Nairobi, Kenia.
04 / 1995:	Samenbetrieb-Spezialist in der „Eastern Africa Bean Research Network (EABRN)" Tagung des Internationalen Zentrums für tropische Landwirtschaft (CIAT) in Zusammenarbeit mit dem landwirtschaftlichen Forschungsinstitut Kenia (KARI).
06 - 08 / 1991:	Teilnahme, "Internationales landwirtschaftliches Praktikum" am Virginia Polytechnic Institut und Staatliche Universität, VA., USA.

(Stand May 2005)

Curriculum Vitae

Personal data

Name:	Theophilus Mwendwa Mutui
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Date and Place of Birth:	21.05.1969, Kitui-Kenya
Nationality:	Kenyan

School and Higher Education

1987 – 1988:	Kitui High School in Kitui, Kenya. University entry Examination: Kenya Advanced Certificate of Education.
1989 – 1992:	Horticultural Science at Egerton University, Njoro, Kenya. Degree: B. Sc. in Horticulture (First Class Honours).
1995 – 1999:	Horticulture (major Floriculture) at the University of Nairobi, Kenya. Degree: Master of Science (M. Sc.) in Horticulture.
Since 04 / 2002:	Ph. D student at University of Hannover, Department of Horticulture.

Work Experience

1993 - 1995:	Technical Representative, East African Seed Company, East Africa.
1997 - 1999:	Agricultural Officer II, Ministry of Agriculture, Government of Kenya.
Since 10 / 1999:	Assistant Lecturer, Moi University, Eldoret, Kenya.

Research Interests

Physiological and molecular effects of phytohormones (cytokinin, ethylene, auxin and GA) on post-harvest quality of ornamental plants.

Honours and Awards

Since 10 / 2001:	DAAD Scholarship for Doctoral Studies at University of Hannover.
10 / 2003:	Certificate of Attendance, “Identities in a changing World: Intercultural Communication:-an Opportunity to solve conflicts” by STUBE and DAAD.
1995 - 1998:	DAAD Scholarship for Masters Studies at University of Nairobi.
04 / 1995:	Seed Industry Resource Person in Eastern Africa Bean Research Network (EABRN) Workshop by International Centre for Tropical Agriculture (CIAT) and Kenya Agricultural Research Institute (KARI).
06 - 08 / 1991:	Participated in, "International Agricultural Internship Program" at Virginia Polytechnic Institute and State University, VA., USA.

(As at May 2005)