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RESEARCH PAPER

Ethylene and flower longevity in *Alstroemeria*: relationship between tepal senescence, abscission and ethylene biosynthesis

Carol Wagstaff[1,4,*], Usawadee Chanasut[1], Frans J. M. Harren[2], Luc-Jan Laarhoven[2], Brian Thomas[3], Hilary J. Rogers[4] and Anthony D. Stead[1,†]

[1] *School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey TW20 0EX, UK*

[2] *Life Science Trace Gas Facility, Department of Molecular and Laser Physics, University of Nijmegen, PO Box 9010, 6500GL, Nijmegen, The Netherlands*

[3] *Plant Genetics and Biotechnology, Warwick HRI, Wellesbourne, Warwickshire CV35 9EF, UK*

[4] *Cardiff School of Biosciences, Main Building, Cardiff University, PO Box 915, Cardiff CF10 3TL, UK*

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Abstract

Senescence of floral organs is broadly divided into two groups: those that exhibit sensitivity to exogenous ethylene and those that do not. Endogenous ethylene production from the former group is via a well-characterized biochemical pathway and is either due to developmental or pollination-induced senescence. Many flowers from the order Liliales are characterized as ethylene-insensitive since they do not appear to produce endogenous ethylene, or respond to exogenous ethylene treatments, however, the majority of cases studied are wilting flowers, rather than those where life is terminated by perianth abscission. The role of ethylene in the senescence and abscission of *Alstroemeria peruviana* cv. Rebecca and cv. Samora tepals was previously unclear, with silver treatments recommended for delaying leaf rather than flower senescence. In the present paper the effects of exogenous ethylene, 2-chloroethylphosphonic acid (CEPA) and silver thiosulphate (STS) treatments on tepal senescence and abscission have been investigated. Results indicate that sensitivity to ethylene develops several days after flower opening such that STS only has a limited ability to delay tepal abscission. Detachment force measurements indicate that cell separation events are initiated after anthesis. Endogenous ethylene production was measured using laser photoacoustics and showed that *Alstroemeria* senesce independently of ethylene production, but that an extremely small amount of ethylene (0.15 nl flower^{-1} h^{-1}) is produced immediately prior to abscission. Investigation of the expression of genes involved in ethylene biosysnthesis by semi-quantitative RT-PCR indicated that transcriptional regulation is likely to be at the level of ACC oxidase, and that the timing of ACC oxidase gene expression is coincident with development of sensitivity to exogenous ethylene.

Key words: Abscission, *Alstroemeria*, chloroethylphosphonic acid, ethylene, photoacoustics, RT-PCR, senescence, sensitivity, silver thiosulphate.

Introduction

Ethylene is the major co-ordinator of senescence in many flowers. The deterioration of the corolla in such species is hastened by exogenous ethylene and senescence is accompanied by increased endogenous ethylene biosynthesis (Nichols, 1977). Following pollination of highly ethylene-sensitive flowers such as carnation, a signal passes from the style to the petals through the ovary (Jones and Woodson, 1997) and initiates a burst of ethylene production. In unpollinated carnation flowers the gynoecium is essential for inducing senescence by generating an initial burst of ethylene which subsequently triggers a second, autocatalytic

* Present address: School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, UK.
† To whom correspondence should be addressed. Fax: +44 (0)1784 470756. E-mail: a.stead@rhul.ac.uk

burst of ethylene in the petals (Shibuya *et al*., 2000). This initiates downstream events in the senescence process such as lipid peroxidation (Leverentz *et al*., 2002) and proteolytic activity (Xu and Hanson, 2000).

In ethylene-sensitive species such as carnation, much interest has been shown in the regulation of senescence through the expression of genes encoding 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and 1-aminocyclopropane oxidase (ACO) enzymes (Kende, 1993). Both are up-regulated transcriptionally as petals age (Woodson *et al*., 1992; Jones, 2003) and transcript levels may, in some species at least, be rapidly up-regulated by pollination, for example, tobacco (Sanchez and Mariani, 2002; Weterings *et al*., 2002), orchids (O'Neill *et al*., 1993; Bui and O'Neill, 1998), and *Petunia* (Clark *et al*., 1997). The evidence therefore suggests that ethylene production may be regulated at the transcriptional level in several species.

Flower deterioration is manifested as petal wilting or colour change in several species (Stead and van Doorn, 1994), whereas in others the perianth abscises (van Doorn and Stead, 1997) with little or no loss of fresh weight. In many species, ethylene appears to play little or no part in controlling petal collapse, for example, in daylily (Lay Yee *et al*., 1992) and iris (Celikel and van Doorn, 1995). However, where the perianth abscises ethylene has, with one exception (Sexton *et al*., 2000), always been shown to be involved in the control of the abscission process. For example, in *Arabidopsis*, ethylene was shown to be essential for normal floral organ abscission (Butenko *et al*., 2003; Patterson and Bleeker, 2004). In species where ethylene has been implicated in the control of floral senescence the use of a pulse treatment of ethylene inhibitors such as silver thiosulphate (STS), norbornadiene or 1-methylcyclopropene (1-MCP) usually prolongs flower longevity and such treatments are useful for increasing the vase life of cut flowers (Serek *et al*., 1995).

In *Alstroemeria* flowers the role of ethylene is unclear, Woltering and van Doorn (1988) reported sensitivity to ethylene to be low and commercial recommendations to use STS are usually related to attempts to prevent leaf yellowing rather than prolonging flower vase life (van Doorn *et al*., 1992). Although the senescence of *Alstroemeria* flowers has been reported elsewhere (Collier, 1997; Wagstaff *et al*., 2001, 2003) there has been no systematic attempt to determine if *Alstroemeria* flowers produce ethylene, nor have the effects of ethylene on flower longevity been fully investigated. The implication is that *Alstroemeria* may be similar to other flowers from the order Liliales, such as *Iris* (Celikel and van Doorn, 1995) and *Hemerocallis* (Lay Yee *et al*., 1992), which are insensitive to ethylene and do not produce the growth regulator during extensive petal collapse. However, *Alstroemeria* petals do not show such extreme petal wilting as either *Iris* or *Hemerocallis*, and termination of vase life is by tepal abscission. Therefore this raises the question of whether ethylene is only involved in abscission in this species, or if it has some additional role to play during senescence. An understanding of the role of ethylene in the control of floral deterioration in *Alstroemeria* has commercial relevance for post-harvest handling, as well as furthering understanding of the regulation of floral senescence and abscission.

Previous attempts by this group to determine ethylene production from *Alstroemeria* flowers and isolated petals using gas chromatography found that production was so low that results were very variable and could only be reproduced reliably when studying the ability of ethylene precursors to induce ethylene production (Stead *et al*., 2003). It was not possible to determine the true levels and the timing of ethylene evolution from *Alstroemeria* flowers, or the stage at which they became sensitive to exogenous ethylene. Consequently, a considerably more sensitive technique of a laser-driven photoacoustic detector has been used to measure real-time ethylene evolution from *Alstroemeria* flowers. This has been used previously to detect ethylene biosynthesis successfully from a variety of flowers (Woltering *et al*., 1988) and floral tissues including styles (Woltering *et al*., 1997) and during pollen tube growth (De Martinis *et al*., 2002). Using this technique it has been possible to follow ethylene production from single flowers in a continuous-flow system over a prolonged period of time.

In this paper, expression profiling of genes encoding the enzymes in the ethylene biosynthesis pathway is presented which has been integrated with physiological and photoacoustic measurements of ethylene production. Data are also presented that enable the role of ethylene to be discussed in relation to another key marker of senescence in *Alstroemeria*, that of proteolytic activity (Wagstaff *et al*., 2002).

Materials and methods

Plant material

Alstroemeria flowers (varieties Rebecca and Samora) were obtained from a commercial nursery (Oak Tree Nursery, Egham, Surrey, UK) and were pulled directly from the rhizome of the stock plants 1–2 d prior to flower opening, i.e. at the same stage as commercial harvest. The cultivar Rebecca has been used throughout this study, with Samora being used for comparison in some experiments. The flowers were transported back to the laboratory dry whereupon the stem ends were placed in water. Individual cymes were cut from each stem and placed in vials of dH_2O for experimental purposes and maintained in a growth room (12 μmol m^{-2} s^{-1}; 16 h photoperiod; 60% RH; 21 °C) for the duration of the vase life. All chemicals were from Sigma, Poole, UK. Stages of development are as described in Wagstaff *et al.* (2001) for Samora and Breeze *et al*. (2004) for Rebecca. Stages have been used in figures where endogenous gene expression or enzyme activity was measured, but days have been used in treatments such as CEPA and STS where the treatment altered the time taken to each morphological stage after harvest. In untreated detached cymes stages 0–7 occur 0, 1, 2, 4, 6, 8, 10, and 12 d after harvest, respectively, in

Rebecca, and stages 1–7 occur 1, 2, 4, 5, 7, 9, and 10 d after harvest, respectively, in Samora flowers.

Treatment with STS or CEPA

STS treatment (4 mM $AgNO_3$:32 mM NaS_2O_3) was for 1 h (longer treatments with STS caused blackening of vegetative structures; Chanasut *et al*., 2003) and flowers were then subsequently placed in distilled water. Ethylene treatments were conducted using chloroethylphosphonic acid solutions (CEPA) on both Samora and Rebecca cymes. 20 replicate cymes were placed into CEPA concentrations ranging from 0.005–500 ppm the day prior to flower opening and then the percentage of perianth segments that had abscised each day was observed (recordings taken up to three times a day). Alternatively, the effect of exposure to CEPA on the force required to detach the tepals was recorded using a DFG-1K digital force meter (Shimpo Europe GmbH, Germany).

Measurement of ethylene production

A laser-driven photoacoustic detector was used to measure ethylene evolution in a continuous flow system. Five flowers, just before opening, were placed individually into glass cuvettes with their cut ends in water containing 1–2 drops of commercial bleach to inhibit bacterial growth. Where appropriate, glass beads were used to reduce the internal void volume. Cuvettes were sealed and a flow of ethylene-free air maintained at a rate of 900 ml h^{-1}. The concentration of ethylene in the atmosphere leaving each cuvette was determined periodically (*c*. every hour) by passing the gas into the photoacoustic cell. At each determination point at least 20 measurements of the ethylene concentration leaving the cuvette were performed. Flowers were left in the cuvettes up until the perianth segments abscised, thus ethylene production from day 0 to day 10 were obtained from the same flowers. In all experiments a background control was subtracted from the test cuvettes comprising of an identical cuvette to the experimental ones, but without the inclusion of a flower.

Expression of ACC synthase and ACC oxidase

Two ACC synthase partial cDNAs were cloned from *Alstroemeria* using degenerate primers based on known ACC synthases in the database (ACSF1: GGCYTSGCHGARAAYCA; ACSR1: GTCGAAYCCBGGRTARTA; ACSR2: GCCCARHGGGTTBGANG). The resultant sequences were used to design primers specific to unique regions of each *Alstroemeria* ACC synthase (*ALSACS1-1* using ALSACSF1: GGCTTGGCTGAGAATCAG and ALSACSR1: ATTGAAGGAAATTGAACCTTAC giving a 204 bp product and *ALSACS2-1* using ALSACSF2: GGCTTGGCTGAGAATCAG and ALSACSR2: GCCAGTGGGTTGGATGG giving a 486 bp product). A similar approach for ACC oxidase using degenerate primers (ACOF: ACCTTCGGSACNAARGT and ACOR: CCRTTGGTKATNACYTC) resulted in one clone being found. Specific primers were designed (ALSOACOF: ACCTTCGGCACAAAGGTGAGC and ALSACOR: GATTACCTCCAGCTGGTCACC) to the clone designated *ALSACO1-1*, giving a 218 bp product. Expression was determined throughout vase life using semi-quantitative RT-PCR as described previously in Wagstaff *et al*. (2002).

cDNA was made from 2.5 μg total RNA (extracted using TriReagent according to the manufacturer's instructions with an additional phenol:chloroform clean-up step and ethanol precipitation) using Promega MMLV H^- reverse transcriptase in a 20 μl synthesis reaction. PCR using specific *Alstroemeria* primers to the ACC and ACO genes of interest was then conducted using 1 μl cDNA as a template. A reaction using a primer set spanning an intron in a β-tubulin gene was used as a control to detect any genomic DNA in the template, and as an internal control for normalizing the RT-PCR data. The number of PCR cycles used was optimized using a pooled sample of cDNA for each primer set so that the products were visible on an ethidium bromide gel, but were below the maximal level of product detectable without saturation. Image analysis using UVP Gel Base Pro (UVP Ltd, Cambridge, UK) of product intensity at each cycle determined the optimal cycle number as one near the middle of the exponential phase of amplification to allow both lower and higher readings to be within the linear phase of the reaction. This ensured that the cycle numbers were below the level of the reaction at which template or enzyme became rate-limiting. The PCR was repeated with individual developmental stages at the optimal cycle number and image analysis performed as above on the resultant gel picture. The PCR for each gene of interest and the control β-tubulin gene was duplicated in independent thermocycler runs from the same cDNA stocks. Data for each gene of interest was then expressed relative to β-tubulin levels for each developmental stage.

Protease activity in STS and ethylene-treated petals

STS treatments were applied to cymes as described above. Ethylene (2 ppm) was injected into sealed containers containing isolated cymes in water at stage 0 and left for 15 h, after which the cymes were returned to the growth room conditions described above. A parallel group of cymes were sealed in a similar container without the addition of ethylene to act as a control. Crude extracts were obtained and total protease activity from Rebecca petals was determined using the method of Wagstaff *et al*. (2002) at stages 0, 3, and 6 of flower development.

Results

Perianth abscission

STS delays time to perianth abscission: Time to abscission in Rebecca flowers held in water was approximately 10 d. Treatment with STS delayed the time to perianth abscission in Rebecca and Samora varieties by approximately 2 d although the addition of exogenous ethylene had no effect on hastening abscission with or without STS treatment (Fig. 1). STS was administered at day 0 (stage 2) in both cases, but ethylene treatment was given at day 0 (Fig. 1A) or day 4 (Fig. 1B).

Response to CEPA treatment: Overall longevity of the control flowers was approximately 96 h greater in Rebecca than Samora. Tepal abscission was accelerated by continuous exposure to low concentrations of CEPA, even concentrations as low as 0.5 ppm resulted in petal abscission occurring before those in water (Fig. 2), whilst those in higher concentrations lost their petals in about half the time compared with those in water. In the highest CEPA concentrations, however, petal development was abnormal with thin, paper-like, petals produced that abscised without fully reflexing. Rebecca and Samora flowers showed the same sensitivity (i.e. tepal abscission commenced after 3 d treatent with CEPA) to concentrations of 50 ppm and 500 ppm CEPA. A low concentration of 5 ppm caused earlier abscission in Samora (10 d) than Rebecca (12 d) and Samora showed sensitivity to even lower CEPA concentrations down to 0.005 ppm. Abscission of petals within each treatment took place over a very short time frame (24 h) showing a high degree of co-ordination and replication.

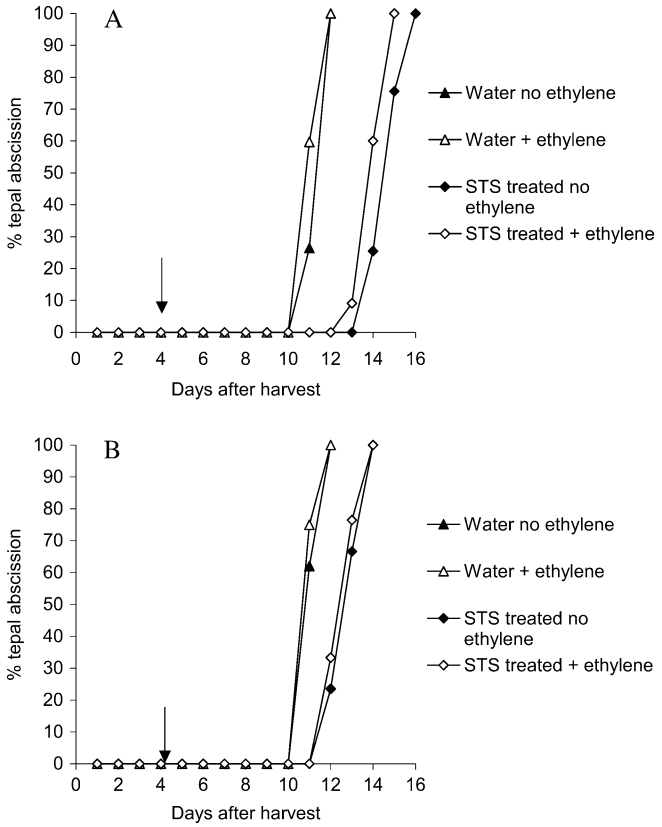

Fig. 1. Tepal abscission in Rebecca following (A) STS (4 mM $AgNO_3$:32 mM NaS_2O_3) treatment at day 0 followed by 2 ppm ethylene for 15 h at day 0 and (B) STS treatment at day 0 followed by 2 ppm ethylene for 15 h at day 4. Arrows indicate the time at which anthesis is complete: n=60 tepals per treatment.

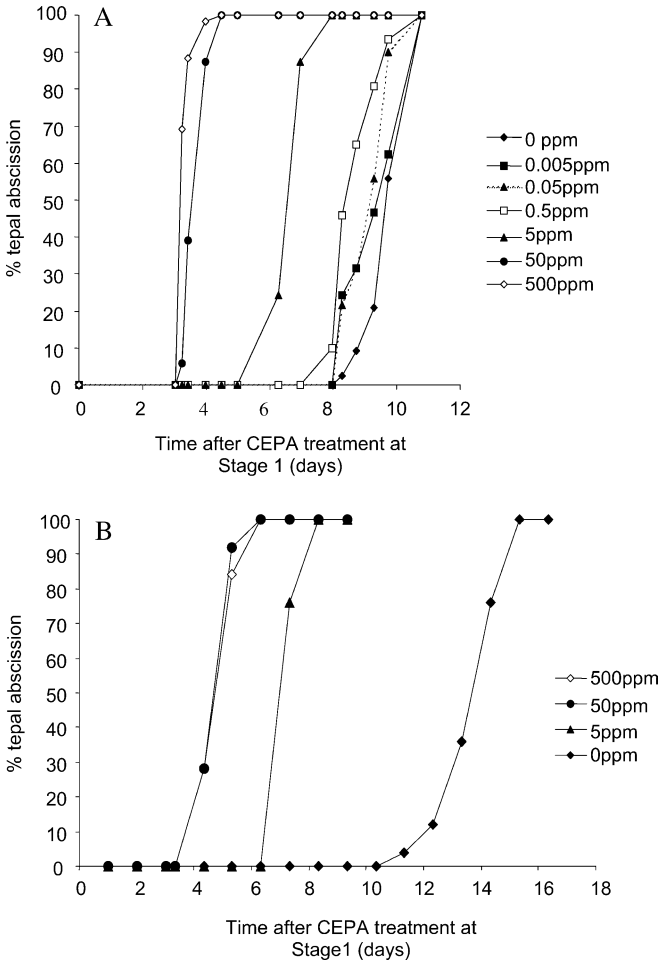

Fig. 2. Tepal abscission (%) for flowers (A) Samora and (B) Rebecca held in varying concentrations of CEPA. n=120 flowers per treatment for Samora: n=150 tepals per treatment for Rebecca.

Detachment force: Flowers from Rebecca were given a range of concentrations of CEPA as shown in Fig. 2 from stage 0 and the force required to detach the petals was subsequently measured at each stage of development (Fig. 3). All concentrations of CEPA tested reduced the force required to detach petals compared with the controls. However, the lower concentrations of CEPA (0.05 ppm and 0.5 ppm) did not reduce the force required to detach the petals until 4 d after the start of the treatment.

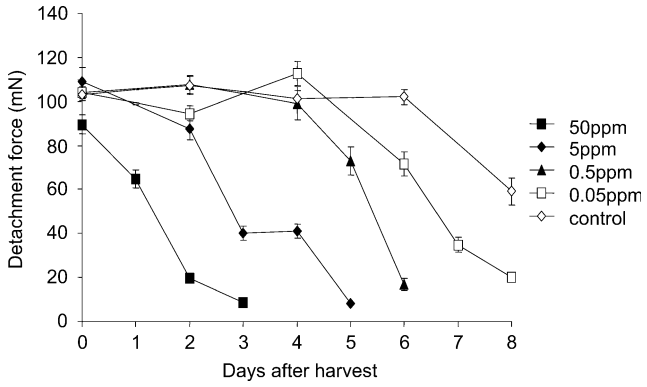

Fig. 3. Detachment force (mN) of Rebecca petals after CEPA treatment: n=30 petals per stage per treatment ±SE.

Ethylene production

Ethylene production from single isolated *Alstroemeria* cv. Rebecca flowers was close to the limits of detection using gas chromatography and no increase in production was detected throughout the life of the flowers. Therefore the extremely sensitive technique of laser photoacoustics was utilized to assay the evolution of ethylene from individual flowers in a continuous flow system.

Ethylene production was detectable from the very youngest flower stages of Samora and Rebecca varieties (when flower fresh weight was approximately 1.5 g) although the amounts produced were very low, less than 0.01 nl flower^{1} h^{-1}. Ethylene production increased about 9 d after harvest in four out of the five Rebecca flowers investigated to a maximum (when the flower fresh weight was approximately 2 g) just less than 7.0 nl flower^{-1} h^{-1}. The fifth flower produced a very small peak of ethylene somewhat earlier (Fig. 4A) and then did not show any

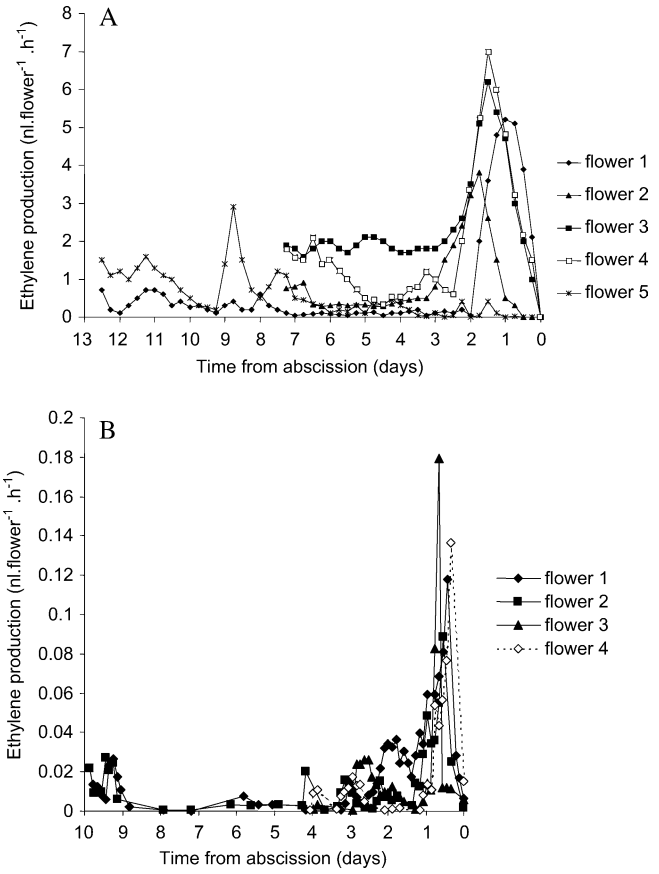

Fig. 4. Ethylene production from isolated *Alstroemeria* flowers (A) Samora (B) Rebecca as detected by photoacoustics. Isolated flowers were held in glass cuvettes with 900 ml h^{-1} of air passing through and the ethylene content of the emitted atmosphere determined at regular intervals throughout vase life.

increased ethylene production when the petals abscised. The increase in ethylene production appears to have taken place over about a 24 h period and the data indicated that this was a transitory burst lasting 24–48 h only. After this burst of ethylene the production seemed to decline at a similar rate to that at which it increased. However, the experiment did not run until the ethylene production had returned to its previous level, as the perianth had abscised and the flower had reached the end of its vase life. A similar pattern of ethylene production was seen in Samora (Fig. 4B) in the 24 h prior to abscission, but the levels produced from this variety were approximately 40-fold lower (on average 0.15 nl $flower^{-1}$ h^{-1}). Ethylene concentration in the atmosphere leaving the flower-containing chamber was between 0.2–0.7 ppb for fully open flowers. Given that the flow rate was 900 ml h^{-1} this equates to less than 1 nl per flower per hour (cv. Rebecca) or less than 0.3 nl g^{-1} FW h^{-1}. This demonstrates the sensitivity of photoacoustics over other ways of measuring ethylene; the ethylene concentration leaving the Samora flower-containing chambers was accurately detected well below 1 ppb.

ACS and ACO expression

ACC synthase and ACC oxidase gene expression was investigated in Rebecca, as this variety showed the greatest production of ethylene. The *ALSACS* and *ALSACO* partial cDNAs isolated showed high homology to the same amino acid regions from a range of other species (alignment not shown) and included conserved amino acids defined in Mita *et al*. (1999). However, they appear to represent two different ACS genes as they shared only 40.6% with one another. In addition *ALSACO1-1* contained all the residues over this region that are essential for ACC oxidase activity of Kiwifruit (Lay *et al*., 1996). The ACC synthase partial cDNAs did not extend to the region encoding the active site of this protein. *ALSACO1-1* (accession no. AY682558) represents a region encoding 73 amino acids starting approximately half way along the full length predicted protein (~317 amino acids). *ALSACS1-1* (accession no. AY682556) and *ALSACS2-1* (accession no. AY682557) begin approximately 50 amino acids from the start of the coding region and encode 107 and 164 amino acids from a predicted full length for ACS proteins of approximately 490 amino acids.

Expression profiles for the three genes were determined by semi-quantitative RT-PCR, visualized by ethidium bromide staining (Fig. 5A). Primers amplifying a portion of a β-tubulin gene spanning an intron were used as a control for genomic DNA contamination. The band strength of the ACS and ACO gene products were quantified by image analysis and normalized to the values obtained for the tubulin cDNA-derived amplicon at each developmental stage (Fig. 5B). *ALSACS1-1* and *ALSACS2-1* showed little change over the course of development and senescence, but *ALSACO1-1* showed marked up-regulation in the last two stages prior to abscission.

Protease activity after STS and ethylene treatment

Protease activity was previously shown to increase dramatically from stage 5 until abscission (Wagstaff *et al*., 2002). Petals from Rebecca flowers treated with either STS or ethylene at stage 0 were assayed for protease activity. Neither treatment significantly changed the level of protease activity at stage 3 compared with the controls, however, STS and ethylene significantly reduced or increased the activity at stage 6 respectively, compared with control levels (Fig. 6).

Discussion

In *Alstroemeria,* petal abscission terminates the functional life of the flower. Although petal wilting is not always associated with increased ethylene production or sensitivity in other species there is only one reported example of non-ethylene inducible petal abscission (Sexton *et al*., 2000). *Alstroemeria* appears to be no exception since a 1 h pulse

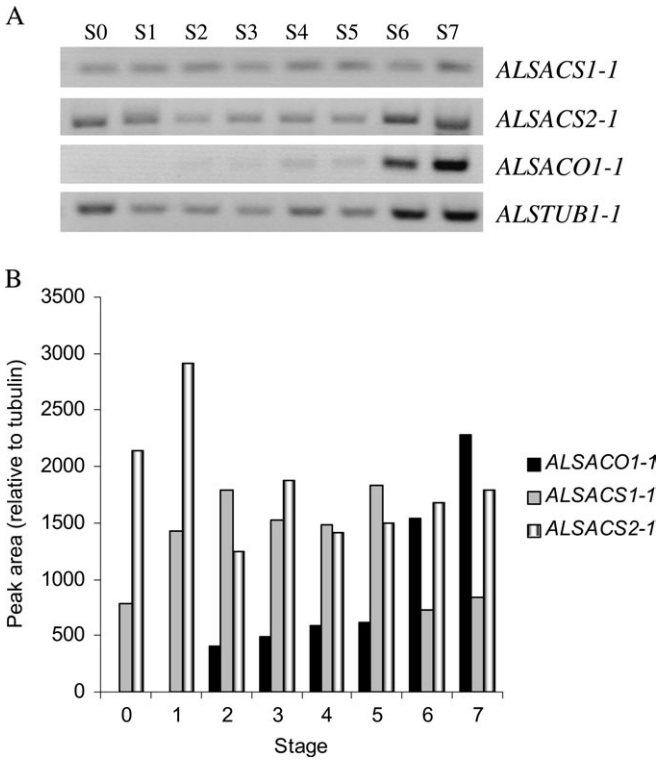

Fig. 5. (A) Expression of two ACC synthase genes and one ACO gene during vase life. Tubulin expression is shown below as a control. All RT-PCR reactions were from the same batch of cDNA and the experiments were repeated at least twice with identical results, only one image of which is shown here. (B) Image analysis of ACC synthase and ACO gene expression profiles shown in part (A), relative to tubulin expression. Stages defined as: S0, harvest point, closed bud; S1, loose bud, sepals parted; S2, open flower, no anthesis; S3, top three anthers anthesed; S4, bottom three anthers anthesed; S5, stigmatic lobes open; S6, sepals translucent at margins, anthers collapsed on bottom petal; S7, perianth abscinds if tapped.

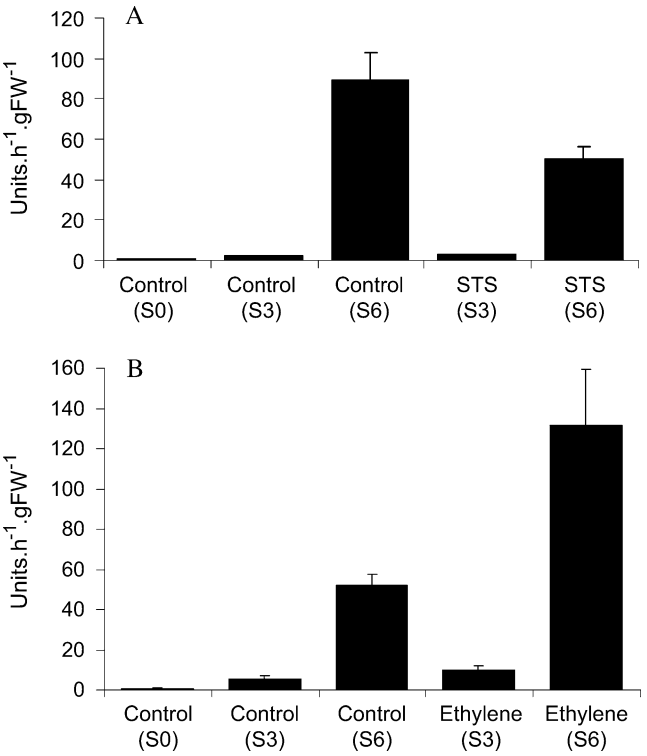

Fig. 6. Protease activity of Rebecca petals following (A) for 1 h and (B) ethylene treatment (2 ppm for 15 h). Treatments administered at stage 0, total protease subsequently measured from crude total protein extract at stage 3 and stage 6 in parallel to controls that had been maintained in water. Data are the mean of three replicates of three independent extractions. Bars=range.

with STS delayed perianth abscission, albeit by only 1–2 d suchthat vase life was 120% that of the controls. This is a small increase compared with the effect STS has on ethylene-sensitive species such as carnation where vase life is extended by 200% compared with untreated flowers (Uda *et al*., 1996). Evidence from ethylene-insensitive transgenics and naturally occurring varieties implies that there is an underlying senescence mechanism that is simply accelerated by the presence of ethylene. For example, *etr1* transgenic petunias show physiologically and morphologically similar senescence to unpollinated non-transformed plants, but at a much later time (Gubrium *et al*., 2000). Similarly, the Chinera variety of carnation shows extremely low levels of endogenous ethylene production compared with varieties such as White Sim, and shows greater longevity (Woltering *et al*., 1993). Therefore it is suggested that senescence is essentially the same in all senescing floral organs, but the sensitivity of some species to ethylene during the senescence phase accelerates this process. This could be said to provide a selective advantage to these flowers following pollination as it provides a rapid mechanism by which to render the flower unattractive to pollinators. In several species, such as lupin, this view is supported by evidence that ethylene production immediately after successful pollination induces a colour change in the petals as a precursor of senescence (Stead and Reid, 1990). This has the immediate effect of dissuading potential pollinators from visiting the flower again.

The ability of STS to delay perianth abscission in *Alstroemeria* suggests that ethylene does play a role in co-ordinating abscission since STS blocks the ethylene receptor (Veen, 1979). Confirmation of this comes from exposing the flowers to ethylene released from CEPA; concentrations as low as 0.5 ppm showed accelerated perianth abscission but at 5 ppm the effect was considerably more noticeable. The flowers were still buds when initially placed in the CEPA solutions, and those in the highest concentration (500 ppm) failed to open properly with abscission of the partially open perianth parts occurring some 3–4 d later, thus CEPA not only induced premature abscission but also prevented normal flower development. At somewhat lower concentrations of CEPA, bud opening was complete, but petals and sepals abscised earlier than water-treated controls, in a concentration-dependent manner. Abscission in all concentrations of CEPA took place over a narrow timeframe of some 24 h suggesting that

ethylene may play a co-ordinating role in this process, as observed in *Rubus* petals (Burdon and Sexton, 1993). However, gaseous ethylene, even when administered as late as day 4 (completion of anthesis), did not accelerate petal abscission (Fig. 1B). This is in contrast to the effect of ethylene on corolla detachability in many other species, for example in *Digitalis*, ethylene exposure caused corolla abscission within 24 h of treatment in all open flowers but not in buds (Stead and Moore, 1983). There is clearly a conflict in the timing of abscission between flowers held continuously in CEPA and those subjected to a pulse of gaseous ethylene at day 0 or day 4. It is hypothesized that ethylene receptors are not functional until after day 4 and that the highest concentrations of CEPA had a toxic effect, as shown by the flowers' deformed morphology which was associated with premature abscission. Such toxicity is not unknown and previous work has suggested that the phosphate in CEPA may account for the morphological deformity seen in olives after treatment with high concentrations of CEPA (Burnik-Tiefengraber *et al*., 1994). The lower concentrations of CEPA accelerated abscission from day 5, presumably after ethylene receptors had begun to become functional. This time point correlates with the stage at which *ALSACO1-1* expression was first detected.

The force required to detach the petals was reduced in CEPA-treated flowers relative to the controls, although for concentrations less than or equal to 0.5 ppm there was no effect on the detachment force prior to day 4 of treatment. This supports the hypothesis above that younger flowers are insensitive to ethylene. Tepal abscission from young flowers treated with high concentrations of CEPA indicates that cell separation around the abscission zone may be relatively early, however, such premature tepal abscission was associated with the deformation of the tepals, presumably because such high concentrations were toxic. The detachment force was reduced in both control and CEPA (0.5 ppm and lower) treated flowers approximately 4 d before abscission occurred. This is considerably later than the ethylene-sensitive abscising species *Arabidopsis*, where cell separation and a decrease in petal detachment force (breakstrength) occurs from the time of anthesis (Patterson and Bleeker, 2004). In *Alstroemeria* flowers maintained in water, anthesis begins 2 d after harvest, but breakstrength does not reduce for another 2 d, i.e. after anthesis of all six anthers has occurred. This time point is coincident with opening of the stigmatic lobes, and is therefore similar to the situation in *Digitalis* (Stead and Moore, 1977).

Despite the ability of CEPA to induce, and for STS to delay, abscission of the perianth the endogenous production of ethylene was very low; indeed no significant differences were detected from isolated flowers of differing ages when gas chromatography was used. Using a laser-driven photoacoustic detector, the detection limit is greatly reduced (Woltering *et al*., 1988), furthermore, this technique uses a flow-through system, thus avoiding the build-up of ethylene in a closed container which might otherwise stimulate the production of autocatalytic ethylene (Lelièvre *et al*., 1998). The low level of ethylene production from both *Alstroemeria* varieties was much less than that associated with other flowers, for example, carnation flowers produced over 80-fold more with a maximum of 25 nl g^{-1} FW h^{-1} (Shibuya *et al*., 2000). In young tomato flowers production is even higher; before pollination a minimum of 150 nl g^{-1} FW h^{-1} was produced, rising to over 300 nl g^{-1} FW h^{-1} after pollination (Llop-Tous *et al*., 2000). In tulips, low levels of ethylene production were reported throughout senescence and during abscission, but uniquely in one of the varieties studied using photoacoustics, tepal abscission appeared to occur in the complete absence of ethylene production (Sexton *et al*., 2000). There was a 4–5-fold increase in ethylene production from *Alstroemeria* flowers concomitant with perianth abscission. An *Alstroemeria* flower weighs a maximum of 2 g, hence this is still equal to only 6 nl g^{-1} FW h^{-1} in Rebecca, much less than reported for other species. This increase occurred over a 24 h period, moreover it would seem that the rate of production declined at a similar rate. Such low rates of ethylene production are consistent with the observation that very low concentrations of CEPA accelerate perianth abscission, thus it would appear that *Alstroemeria* flowers might be very sensitive to ethylene, but sensitivity only develops late in vase life. Indeed perhaps it is this very sensitivity, and the timing of it, that prevents STS from having such a dramatic effect on flower longevity in this species.

Given that there was an increase in the rate of ethylene production from isolated *Alstroemeria* flowers, it was anticipated that the genes involved in ethylene biosynthesis might show up-regulation in the later stages of petal senescence. However, the normalized values of *ALSACS1-1* and *ALSACS2-1* showed a less than 2.5-fold change from S0 to S7, indicating that these members of the ACC synthase gene family are unlikely to regulate ethylene biosynthesis transcriptionally. This is in contrast to some flower species, including Rose varieties such as Kardinal (Wang *et al*., 2004). It is possible, however, that transcriptional regulation of ethylene biosynthesis is at the ACC oxidase level since *ALSACO1-1* showed very little change over the first six stages of development, but then increased by over 4.5-fold in the last two stages of development prior to abscission. In addition, previous work has shown that *Alstroemeria* petals can convert >1 mM ACC to ethylene, supporting the conclusion that ACC oxidase is functional in this system (Stead *et al*., 2003). A similar pattern of gene expression was observed in the rose cultivar Bronze, which showed no change in ACC synthase expression during senescence, but an increase of an ACC oxidase transcript prior to petal abscission (Muller *et al*., 2000). Thus in rose the transcriptional regulation of ethylene biosynthesis may be variety-specific. In a project related to the present one, nearly 2000 *Alstroemeria* transcripts were sequenced from

subtracted (Breeze *et al*., 2004) and global (C Wagstaff, unpublished data) cDNA libraries in order to identify both up- and down-regulated genes at several stages between stage 0 and stage 5. Of these, none relating to ethylene biosynthesis were identified, supporting this hypothesis that ethylene biosynthesis only occurs after this stage, i.e. post-stigmatic lobe opening. Such patterns of expression show that ethylene biosynthetic genes are up-regulated very late on, in fact after those processes that had previously been identified as being up-regulated around stage 5: for example, DNA laddering, electrolyte leakage, fresh weight loss, and protease activity. By contrast, sensitivity to applied ethylene develops a little earlier, probably concomitant with these processes (Fig. 7c in Wagstaff *et al*, 2003).

From measurements of total protease activity it was concluded that acceleration of abscission by exogenous ethylene results in a concomitant acceleration of protein breakdown. Conversely, delaying abscission with STS results in reduced levels of protease activity. Thus it is clear from these data that physiological markers of senescence other than abscission are regulated by ethylene in this species. Exogenous ethylene application has been shown to enhance protease activity in parsley leaves (Jiang *et al*., 1999) and cysteine protease gene expression in carnation petals (Jones *et al*., 1995), resulting in premature senescence. It is not certain from the present study if changes in endogenous levels of ethylene are a precursor of proteolytic activity. From previous studies (Wagstaff *et al*., 2002) the reverse would appear to be the case as a rise in proteolysis was observed from stage 5 onwards. This is several days ahead of the rise in ethylene production reported in the present work, but concomitant with the time at which *ALSACO1-1* expression was detected. Therefore it was hypothesized that proteolysis can be induced or repressed by altering ethylene levels, but that in the absence of external STS or ethylene treatments the proteolytic cascade is initiated and regulated by some other means.

An examination of the ethylene sensitivity and responsiveness of *Alstroemeria* flowers using a range of techniques has shown that this species senesces independently of endogenous ethylene production, but that the completion of abscission requires a small burst of ethylene in the previous 24 h prior to cell separation. The use of photoacoustics has enabled the endogenous ethylene production of this species to be characterized very precisely, and in a way that would not have been possible with older technologies. Using a gas chromatograph this species would be classified as showing ethylene-insensitive abscission, a conclusion that would have been incorrect, since *Alstroemeria* is clearly sensitive to extremely small concentrations of endogenous and exogenous ethylene, although sensitivity develops late in the life of this flower. In addition, the photoacoustic data supported evidence from abscission following CEPA treatment that there is a varietal difference in the sensitivity of *Alstroemeria* to ethylene. Samora responded to lower concentrations of CEPA than Rebecca and produced an order of magnitude less ethylene than the latter. Alternative systems of measurement using static gas collection systems rather than a continuous flow method raise questions of autocatalytic ethylene production in sealed chambers such that it is not always clear exactly what is being measured. Photoacoustics is a valuable tool with potential for examining other ethylene-producing events in plant development such as wound- and pollination-responses at a much more sensitive level than has previously been possible, and may lead to the recharacterization of processes in some species that were thought to be ethylene independent.

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