Ethylene and carbon dioxide production by developing strawberries show a correlative pattern that is indicative of ripening climacteric fruit

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Laser photoacoustic spectroscopy continuously quantified the ethylene (C\textsubscript{2}H\textsubscript{4}) produced by strawberry flowers and fruits developing in planta. C\textsubscript{2}H\textsubscript{4} was first detected as flower buds opened and exhibited diurnal oscillations (to approximately 200 pl flower\textsuperscript{−1} h\textsuperscript{−1}) before petal abscission. Exogenous application of silver thiosulphate (STS) to detached flowers inhibited petal abscission and flower senescence. In fruit, C\textsubscript{2}H\textsubscript{4} production was maintained at a ‘low level’ (10–60 pl fruit\textsuperscript{−1} h\textsuperscript{−1}) until fruit expanded when levels increased in a diurnal pattern (to 200 pl fruit\textsuperscript{−1} h\textsuperscript{−1}). After expansion, C\textsubscript{2}H\textsubscript{4} production declined to a low level until fruit attained the red-ripe stage for at least 24 h. After this time, C\textsubscript{2}H\textsubscript{4} levels increased linearly (no diurnal fluctuation) to approximately 1 nL fruit\textsuperscript{−1} h\textsuperscript{−1}. Twenty-four hours after the re-initiation of C\textsubscript{2}H\textsubscript{4} production by red fruit, CO\textsubscript{2} levels increased approximately three-fold, indicative of a respiratory climacteric. STS applied to fruits developing in planta and dissected fruit parts ex situ established that C\textsubscript{2}H\textsubscript{4} production is regulated by negative feedback until fruits had expanded. The C\textsubscript{2}H\textsubscript{4} produced by red-ripe fruit was regulated by positive feedback. Anti-1-amino-cyclopropane-1-carboxylic acid oxidase IgG localization identified immunoreactive antigens of 40 and 30 kDa (M\textsubscript{r}) within the fruit achenes of expanding and red-ripe fruit. Analysis of dissected fruit showed that seed C\textsubscript{2}H\textsubscript{4} accounts for 50% the C\textsubscript{2}H\textsubscript{4} that is detectable from ripe fruit.

Introduction

Strawberry (\textit{Fragaria}) fruits differ from most other fruits in numerous ways (Perkins-Veazie et al. 1996). Strawberries are one of the few fruits derived from receptacle (non-ovarian) tissue, whereas ‘true’ strawberry fruits (the achenes) are situated on the outside of the berry epidermis. Achene-derived auxin is the key phytohormone controlling the growth and ripening of strawberry receptacles (Nitsch 1950, Given et al. 1988, Medina-Escobar et al. 1997). To some extent, this finding has encouraged a reinforcement of the contentious concept that fruit ripening in strawberry is a non-climacteric and an ethylene (C\textsubscript{2}H\textsubscript{4})-insensitive process (Rhodes 1997).

Abbreviations – 1-MCP, 1-methyl cyclopropene; ACC, 1-aminocyclopropane-1-carboxylic acid; DS, developmental stage; LPD, laser-based photoacoustic trace gas detector; SE-IgL, silver-enhanced immunogold labelling; STS, silver thiosulphate.
Classically, the definition of a climacteric fruit has three distinct component parts: (1) an autocatalytic increase in \( \text{C}_2\text{H}_4 \) production; (2) an associated increase in respiration, which is referred to as the ‘respiratory climacteric’; and (3) that (1) and (2) are accompanied by phenotypic (and genetic) changes in the fruit that lead them to be identified as ripe (Rhodes 1970). In this article, we investigate attached strawberry fruit ripening in planta and determine whether this ripening process is climacteric, particularly because the first component of the climacteric definition makes no claim regarding the absolute quantities of gaseous \( \text{C}_2\text{H}_4 \) that are produced, nor the response of fruit to inhibitors of \( \text{C}_2\text{H}_4 \) production or reception.

The 1-aminocyclopropane-1-carboxylic acid (ACC)-dependent biosynthesis of \( \text{C}_2\text{H}_4 \) has been found, without exception, in all higher plant species systems studied (Yang and Hoffman 1984), and ripe, as opposed to immature or developing strawberry fruit, may be no exception to this characteristic. However, the rate of \( \text{C}_2\text{H}_4 \) evolved from ripe strawberry fruit is 20–2000 times lower than many other so-called \( \text{C}_2\text{H}_4 \)-responsive or climacteric fruits; the application of exogenous \( \text{C}_2\text{H}_4 \) to mature detached green fruits does not promote strawberry fruit-ripening processes (Perkins-Veazie et al. 1996). On the other hand, the expression of cystathionine-gamma-synthase, an enzyme that is involved in the synthesis of methionine (a \( \text{C}_2\text{H}_4 \) precursor), is highly upregulated with the onset of strawberry ripening and is also repressed by auxin (Marty et al. 2000). The higher levels of \( \text{C}_2\text{H}_4 \) generated by harvested ripe strawberries also play a causal role in post-harvest decay processes (Tian et al. 1997). Furthermore, the positive qualities of just-ripe strawberry fruit, such as fruit firmness and depth of colour, can be extended using chemicals that inhibit \( \text{C}_2\text{H}_4 \) action, such as potassium permanganate (Wills and Kim 1995, Kim and Wills 1998) and 1-methyl cyclopropene (1-MCP) (Ku et al. 1999, Sisler and Serek 1999) that slow the rate of anthocyanin and phenolic accumulation (Jiang et al. 2001). It has also been demonstrated that expanded white strawberry fruits supplemented with the endogenous \( \text{C}_2\text{H}_4 \) precursor ACC and ripened in vitro had higher rates of \( \text{C}_2\text{H}_4 \) production, grew to a larger size and exhibited enhanced colour development when compared with untreated fruit (Perkins-Veazie et al. 1995, 1996). These reports are supported by the study of Castillejo et al. (2004) who identified a gene family that encodes a key ripening-specific cell-wall-degrading enzyme, pectin methyl esterase. One of the members of this family (FaPE1) possesses a \( \text{C}_2\text{H}_4 \)-responsive element which might allow specific regulation of expression by the increased levels of \( \text{C}_2\text{H}_4 \) in ripe strawberry fruit. Similarly, evidence has been gathered from other so-called non-climacteric fruit (Biale and Young 1981, Giovannoni 2001) such as oranges and grapes that also show a causal role for \( \text{C}_2\text{H}_4 \) in fruit maturation (Alonso et al. 1995, Chervin et al. 2004, Tesniere et al. 2004).

Although the onset of enhanced \( \text{C}_2\text{H}_4 \) production by red-ripe strawberry fruit has been shown, this phenomenon has not been associated with a respiratory climacteric, even though modified atmosphere packaging physiologists have acknowledged that ripe strawberry fruit possess a very high respiratory rate (Day 1993). As well, other contrasts between fruits such as tomatoes that show an obvious climacteric and strawberry include ‘\( \text{C}_2\text{H}_4 \) self-regulation’, which in climacteric fruit is under negative-feedback control until fruit are fully expanded and pale green in coloration, after which point regulation becomes positive (Mordy et al. 2000). This corresponds to the terms coined, respectively, as \( \text{C}_2\text{H}_4 \) production system 1 and system 2 (Yang and Hoffman 1984). In contrast, the biosynthesis of \( \text{C}_2\text{H}_4 \) in ripening strawberry fruits is reported to be regulated negatively throughout growth and ripening (Perkins-Veazie et al. 1995, Tian et al. 1997, Mordy et al. 2000).

Taken together, these findings have encouraged the general perception that ripe strawberry fruits can be discriminated from other fruits such as tomato and melon on the basis that they are ‘non-climacteric’ (Biale and Young 1981). It should be acknowledged that these claims are based on data gathered from detached fruit, which in some instances were ripened in vitro. In addition, this concept of strawberry fruit ripening has drawn heavily on conclusions extrapolated from metabolite or enzyme activity data. With either of these approaches, there is a risk that the measurements collated do not reflect the ripening processes that occur in planta. This may be due to the removal of important growth promoters as a result of fruit detachment and/or dissection or due to the release of components in fruit extracts that inhibit enzyme activities or bind protein or metabolites (Iannetta et al. 2004, Souleyre et al. 2004).

Consequently, we used a state-of-the-art trace gas photoacoustic detector to detect very low levels of \( \text{C}_2\text{H}_4 \). The instrument, a laser-based photoacoustic trace gas detector (LPD), exploits infrared spectroscopy (Bijnen et al. 1996, Thain et al. 2004) to accurately detect \( \text{C}_2\text{H}_4 \) at very low concentrations (down to 0.01 ppbv = 0.01 part per billion volume). This was used to continuously monitor \( \text{C}_2\text{H}_4 \) as it is evolved from strawberry fruits as they matured in planta from the closed flower bud stage to the late red-ripe stage. This involved the design of specific apparatus and particular measurement parameters that are detailed. To assess the potential relationship between \( \text{C}_2\text{H}_4 \) production and fruit
respiration, we carried out ‘in-line’ measurements of fruit CO₂ production on berries ripening in planta. The unique approach we employed generated very interesting and novel data, the nature of which raised questions as to the regulation and source of the C₂H₄ production. Consequent studies involved the administration of C₂H₄ reception inhibitors to flowers and fruits at various developmental stages (DSs), and the resultant effects on C₂H₄ production and regulation were assessed. Additional investigations to identify the relative contribution of the component fruit parts were also carried out by measuring C₂H₄ production from dissected fruit tissues.

**Materials and methods**

**Plant material and growth conditions**

Fruiting strawberry plants (Fragaria × ananassa Duch. cultivar Elsanta) in 60 × 30 cm pots (six pots with six plants per pot; 36 plants in total) were obtained from local producers based on the outskirts of Nijmegen (the Netherlands). Plants were taken from a raised-bed, polythene-tunnel field-based cultivation system and transferred to a glasshouse at the University of Nijmegen where they were subject to a natural light and temperature regime. Selected plants were transferred to the laboratory where culture conditions were 16 h of daylight level of 300 μmol m⁻² s⁻¹ and a constant temperature of 20°C for the duration of the measurement period that ranged from 24 to 240 h. Physiology measurements were carried out on flowers and fruit. The flowering stages included closed flower buds and open flowers that appeared healthy with all petals still attached and showing no visible signs of senescence. Six different fruit DSs were used and are defined as DS1, DS2 and DS3 (approximately 7, 14 and 21 days after anthesis, respectively) representing small dark-green fruit of increasing size with tightly packed achenes; DS4, representing expanded (achenes no longer tightly packed) pale-green fruit; DS5, representing fruit at the reddening stage (often referred to as pink fruit) and DS6, representing fruit that has attained a red-ripe phenotype for a period of at least approximately 24 h and is not evolving C₂H₄ as determined by LPD determination. Beyond this time, a further stage denoted as DS6/24 h⁺ was identified as a consequence of our LPD measurements and is defined as ripe fruit that has attained DS6 for a period of 24 h or more when C₂H₄ production and re-initiated.

**C₂H₄ and CO₂ quantification**

The flowering or ripening stages defined above were enclosed in purposely designed glass cuvettes schematically represented in (Fig. 1A). A seal to contain accumulated gases was facilitated as follows. Non-C₂H₄-evolving cylindrical and tapered rubber stoppers were prepared to accommodate flower or fruit stalks by first drilling a hole longitudinally through the centre of the stopper. The stopper was then half-sliced from the outer edge to the centre-hole, which was sufficiently large to avoid stem crushing when the stopper was re-closed. The stopper was then positioned into the neck of the cuvette. Occasionally, it was necessary to remove the sub-tending leaflets and fruits. Cuvettes were held in place using clamps supported on clamp stands (Fig. 1B). A complete gaseous seal was maintained by packing Terostat™ (Terostat-IX, Henkel-Teroson, Heidelberg, Germany) around the stem and stopper. Non-C₂H₄-producing nylon tubing (22-NF-1/8, Imperial-Eastman, Baltimore, MD) was attached to the arms of each cuvette for the carrier gas (air) in/out flow. Flowers or fruits were enclosed in glass cuvettes, and evolved gas was allowed to accumulate for 2 h before C₂H₄ measurement. Measurements from detached fruit were made with a 2.3-h accumulation time. In between these stops, the gas flow rate was maintained at 1 l h⁻¹ for all measurements (Thain et al. 2004). Fig. 1B shows the containment cuvettes used in the growth cabinet, with a series of fruits ripening from DS4 to DS6/24 h⁺.

Two control treatments were included consisting of either a sealed empty cuvette or a cuvette enclosing stems whose leaflets and fruits or flowers had been removed to simulate the damage that may have been incurred to the fruit-bearing stem as part of the fruit enclosure process. In each case, C₂H₄ levels in the control cuvettes remained below the detectable limits of the LPD instrumentation. There was a possibility that CO₂ accumulated in cuvettes during the 2-h interval between C₂H₄ determinations would influence C₂H₄, particularly for the high-respiring ripe fruit. However, fruits allowed to ripen in a constant flow of CO₂-enriched air produced levels of C₂H₄ that were comparable with those of control fruits that ripened in a continuous flow of CO₂-free air. In addition, fruits enclosed in an atmosphere artificially enriched by the addition of exogenous CO₂ showed levels of C₂H₄ accumulation that were not significantly different from those of control fruit. Note also that the number of diurnal C₂H₄ peaks varied depending on the individual flower or fruit being studied; this requires further investigation but is possibly related to organ size with larger flowers and/or fruit producing equivalent absolute quantities of C₂H₄ for longer periods. Consequently, the chosen data reflect
individuals that produced the same number of diurnal cycles for adequate replication and standard error measurements.

The production of CO$_2$ and C$_2$H$_4$ was measured in parallel from five different attached fruits at DS4 (ripening to DS6/24 h+) along with one empty control cuvette. A carrier gas flow rate of 1.7 l h$^{-1}$ was maintained for an 8-day period during which time fruit gas production was monitored every 20 min approximately. The gas that was produced passed through an infrared gas analyser for CO$_2$ detection (URAS 14, Hartmann & Braun, Frankfurt, Germany) that was interfaced to a personal computer before passing to the LPD chamber for C$_2$H$_4$ determination.

**Application of STS to flowers and ripening fruit of DS3 to DS6/24 h+**

Stock solutions of 0.1 M sodium thiosulphate and 0.1 M silver nitrate were prepared and stored separately in the dark. A solution of 10 mM silver thiosulphate (STS) was prepared by slowly combining one volume of silver nitrate with a (stirring) solution of nine volumes of sodium thiosulphate. Experiments on attached fruit samples were performed by injecting 50 µl of STS solution directly into the pedicel vasculature at the base of each flower or fruit-ripening stage using a fine-needled 702 Hamilton syringe (Hamilton Company, Bonaduz, Switzerland). Control fruit were injected with 50 µl of
distilled water. Detached tissues were exposed to STS by placing the cut stems directly into the solution. $\text{C}_2\text{H}_4$ production in response to STS application was monitored from approximately 3 h after injection as described above.

Quantification of $\text{C}_2\text{H}_4$ evolved from dissected fruit tissue

To identify the source of $\text{C}_2\text{H}_4$ production, we made measurements on dissected fruit parts of DS4–6/24 h+. Four tissue types were assessed: (1) pooled achenes – removed using fine forceps to minimize damage; (2) de-achened fruit ‘peels’ – removed to a depth of approximately 3 mm using a sharp razor blade; (3) achened epidermis – peels collected as described in (2) but including the achenes and (4) damaged achened epidermal peels – punctured once for each achene that the peel possessed using the point of fine forceps to mimic the maximum physical damage caused by achene removal. Note that equivalent proportions of the whole strawberry tissue were used. That is, the area of each epidermis that was measured possessed the same number of achenes. Also, the number of achenes used to measure $\text{C}_2\text{H}_4$ of ‘achen-only’ samples were pooled from that same area of epidermis. To avoid dehydration, we placed each tissue type onto a bed of water-saturated filter paper circles (595 S&S, Schleicher and Schuell, Dassel, Germany) placed into a horizontally held 20-mI glass vials (crimp top vials, Varian $^\text{TM}$, Den Bosch, the Netherlands) and sealed using Varian $^\text{TM}$ crimp tops. Control flasks comprised either empty cuvettes or cuvettes that contained only water-saturated filter paper. $\text{C}_2\text{H}_4$ was monitored as described above, and data obtained were standardized using the fresh weight of tissue tested to give $\text{C}_2\text{H}_4$ production rates as $\text{pl h}^{-1}$ $\text{g}^{-1}$ FW.

Western analysis and silver-enhanced immunogold labelling (SE-IgL) of fruit proteins

Freshly harvested strawberry fruits that had attained DS6/24 h+ were quickly dissected (where necessary) and frozen in liquid nitrogen. The frozen samples comprised (1) *Trifolium repens* (white clover) as a positive control, (2) fruit epidermal peel plus achenes, (3) achenes only, (4) de-achened epidermal peel, (5) fruit flesh only (minus achenes, epidermis and vascular core), (6) fruit vascular core only, (7) sepals only and (8) whole fruit. Each tissue type was ground to a fine powder under liquid nitrogen using a mortar and pestle and then was weighed into a prechilled 2-ml microfuge tube. Freshly prepared extraction buffer, consisting of 8 M urea, 0.2 M Tris–HCl (pH 8.0), 8.0 mM dithiothreitol and 2% SDS, was added at a ratio of 2:1 (v/w). The sample was mixed by vortexing and incubated on ice for 2 min before centrifugation at 4°C for 10 min at 13 000 g. The supernatant was removed and processed immediately with the addition of (3:1) with 0.005% (w/v) bromophenol blue in sterile glycerol 40% (v/v) with sterile distilled water buffer. Each 20 $\mu$l protein sample was then boiled for 10 min, cooled briefly and the volume pooled by a short (5 s) centrifugation. The proteins were resolved in a 10% Bis-Tris NuPage $^\text{TM}$ denaturing polyacrylamide gel (NP0321BOX; Invitrogen Ltd, Paisley, UK) in a NuPage $^\text{TM}$ MOPS running buffer according to the provider’s instructions. The resolved proteins were subsequently electroblottedted to nitrocellulose at 100 V for 1 h using MiniProtean$^\text{TM}$ equipment (Biorad$^\text{TM}$, Hemel Hempstead, Hertfordshire, UK) according to the manufacturer’s instructions.

For Western analysis, the nitrocellulose membrane was washed immediately after blotting by gentle shaking in 50 ml $\times$1 wash buffer $\times$1 Tris-buffered saline (TBS): 50 mM Tris, 150 mM NaCl, pH 7.6 with 0.01% (v/v) Tween®20 (P1379; Sigma-Aldrich, Poole, UK) for 20 min ($\times$3). The membrane was then immersed in 20 ml blocking buffer [wash buffer with 3% (w/v) bovine serum albumin (BSA) (A2153; Sigma–Aldrich), 2% (w/v) dried milk powder (Marvel®), 0.01% (v/v) Tween®20 (P1379; Sigma–Aldrich)] and incubated for 1 h with gentle mixing. This was decanted and replaced with a fresh 20 ml volume of blocking buffer to which 20 $\mu$l of primary IgG [anti-ACC oxidase (ACO) IgG from white clover; Hunter et al. 1999] was added. This was incubated for a further 1 h with gentle mixing. The blot was washed as described above before a further 20 ml of fresh blocking buffer was re-introduced, to which was added 10 $\mu$l of goat anti-rabbit alkaline-phosphatase conjugate (R5131; Sigma–Aldrich). After a further 1 h incubation, the blot was washed as before ($\times$3) followed by a further five washes of $\times$1 TBS only (20 min, $\times$3) and sterile distilled water (10 min, $\times$2). IgG localization was revealed by incubating the blot in 25 ml of Bio-Rad Laboratories (Hemel Hempstead) liquid substrate system for alkaline phosphatase (170-6432; according to their instructions). Immunoreactive proteins were visualized as dark blue-purple bands.

SE-IgL was carried out on fruit epidermis and excised achenes at DS3–6/24 h+. Harvested fruit were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4), dehydrated in an ethanol series and embedded in LR White acrylic resin. Semi-thin sections (1 $\mu$m) were taken using a Reichert Ultracut E ultramicrotome and collected on glass slides (Superfrost; VWR Ltd, Poole, Dorset, UK), and the sections were immediately processed for SE-IgL according to the methods of James and Sprent (1999) with modifications. After blocking in a buffer containing 1%
Tween-20 and 1% BSA (‘IGL buffer’), the sections were incubated in a 1:100 dilution (in IGL buffer) of the anti-white clover ACO IgG for 1 h, washed and then incubated in a 1:50 dilution (in IGL buffer) of 8-nm gold particles conjugated to protein A for 45 min. After thorough washing, silver enhancement of the sections was performed using the Amersham IntenseM kit according to the manufacturer’s instructions. The following negative controls were used for the immunogold procedure: (1) omission of the primary antibody and (2) substitution of the primary antibody by non-immune sera diluted appropriately in IGL buffer.

Results

C₂H₄ production by ripening strawberry flowers and fruits

All the fruit DSs that were monitored, from the closed flower bud to the most mature red-ripe fruit stages, produced C₂H₄. Flowers started to produce detectable levels of C₂H₄ less than 24 h before bud opening. This was produced in a diurnal pattern and peaked during the daylight hours (Fig. 2A). Petal abscission took place approximately 24 h following a maximal peak in C₂H₄ production (approximately 225 pl flower⁻¹ h⁻¹; Fig. 2A). Experiments, in vitro, with cut open flowers or flower buds cultured with the cut end of the pedicel in either water or STS showed that flower petal abscission was inhibited by STS (Fig. 2B, C). After petal abscission, as spent flowers developed to DS1, C₂H₄ levels were maintained for a prolonged period at the lower detection limit of the LPD (Fig. 2A; 70 h+, 10–60 pl fruit⁻¹ h⁻¹) until late DS3 (to 24 h; Fig. 3A). Elevated diurnal C₂H₄ production was then witnessed with peaks of up to approximately 300 pl fruit⁻¹ h⁻¹ as fruit developed to DS4 (expanded pale-green fruit; 96–120 h; Fig. 3A). C₂H₄ production then fell to the lowest detection limit.

Fig. 2. (A) Ethylene production by flower buds as they mature to open flowers in vivo and as they develop towards developmental stage 1 (DS1). Grey bars depict periods of darkness. The response of detached flower buds and open flowers cultured in vitro with their cut pedicels immersed in either water (B) or 10 mM silver thiosulphate (C).
threshold again as fruit entered DS5 (pink fruit) and until approximately 24 h at DS6 (red fruit, approximately 160 h; Fig. 3A). During all the periods of low C$_2$H$_4$ production (DS1–3 and DS5–6), continuous diurnal cycling with daytime maxima was clearly observed. Between the production minima, dramatic and highly reproducible patterns in C$_2$H$_4$ levels were observed at the flowering stages and DS3–4. C$_2$H$_4$ production from fruit at DS6/24 h+ (Figs 2A and 3A) increased continuously, and without diurnal variation, to levels greater than 500 pl fruit$^{-1}$ h$^{-1}$ (Fig. 3A), although maxima up to of 2 nl$^{-1}$ fruit$^{-1}$ h$^{-1}$ were measured depending on the particular fruit. Data collected from detached fruit at DS4–6/24 h+ showed a very similar pattern to C$_2$H$_4$ production found for attached fruit (data not shown), although, for detached fruit, the decline in C$_2$H$_4$ levels during DS5 was transitory and was not maintained for the 24-h (or more) plateau that is evident in the attached fruit.

**Respiratory measurements and associated C$_2$H$_4$ production**

The increased rate of C$_2$H$_4$ production after DS6/24 h+ was accompanied by a marked elevation in CO$_2$ emission (Fig. 3B). Respiratory activity, as indicated by CO$_2$ production, began to increase relatively slowly, as fruit reached the final stages of DS4 (after 45 h of recording; Fig. 3B), with a production rate of approximately 3 vpm berry h$^{-1}$ h$^{-1}$ until approximately 135 h. This rate increased dramatically only a short time (approximately 1–2 h) after C$_2$H$_4$ levels rose above ‘background’ concentrations (after approximately 135 h of data recording; Fig. 3B). The CO$_2$ levels had doubled 12 h after
$\text{C}_2\text{H}_4$ production had resumed at DS6 + 24 h, representing a rate increase of almost 20 times (to over 50 vpm berry h$^{-1}$ h$^{-1}$). A diurnal cycle in CO$_2$ production was also obvious as indicated by regular maximum and minimum plateaux in the quantities detected. Before fruit reddening, the levels of CO$_2$ production during the night period exceeded daytime levels. However, this difference declined in magnitude until just before the onset of fruit anthocyanin pigmentation (DS5 at approximately 80 h; Fig. 3B), and thereafter, a reverse pattern was observed as in which CO$_2$ production during the night period fell below daytime levels (Fig. 3B). By 24 h after fruit reddening, the respiratory decline becomes more asynchronous, occurring before the dark period is reached. The reason for this is not clear at this time.

**Microinjection of STS**

We tested the possibility that strawberry fruit seeds (or ‘true fruits’) produce significant amounts of $\text{C}_2\text{H}_4$ as compared with the fruit flesh by accessing the cells within the seed via their connection to the mother plant (i.e. by microinjection into the vasculature). Water delivered into the pedicels of flowers disrupted the normal diurnal pattern of $\text{C}_2\text{H}_4$ production (Fig. 4A), with levels remaining stationary throughout the approximately 70-h monitoring period (at approximately 50 pl flower$^{-1}$ h$^{-1}$; Fig. 4A). Similarly, diurnal $\text{C}_2\text{H}_4$ production was halted in flowers injected with STS. It is therefore unlikely that the loss of diurnal $\text{C}_2\text{H}_4$ production is not likely to be due to STS and is a consequence of microinjection also evident for the other DSs that normally exhibit cyclic $\text{C}_2\text{H}_4$ evolution.

Silver treatment had the additional effect of increasing $\text{C}_2\text{H}_4$ production above that seen for the water-injected control flowers. STS-treated flowers reached a peak (250 ± 16 pl flower$^{-1}$ h$^{-1}$; Fig. 4A) 15 h after injection. Thereafter, $\text{C}_2\text{H}_4$ levels declined, and by 30 h, $\text{C}_2\text{H}_4$ production rates attained a plateau that was higher and approximately double that of control flowers (approximately 100 pl flower$^{-1}$ h$^{-1}$; Fig. 4A). Fruit at DS3 also displayed a similar pattern of $\text{C}_2\text{H}_4$ production in response to STS. Control (water injected) fruit at DS3 displayed a pattern of $\text{C}_2\text{H}_4$ production that was low and stationary throughout the approximately 60-h monitoring period (approximately 60 pl fruit$^{-1}$ h$^{-1}$; Fig. 4B). On the injection of STS, $\text{C}_2\text{H}_4$ production increased in a linear fashion attaining a plateau of approximately 160 pl fruit$^{-1}$ h$^{-1}$ after 40 h (Fig. 4A). $\text{C}_2\text{H}_4$ continued to be produced at this elevated level for the following 20 h of measurement. During this later time period, production rates were approximately three times higher than those values (60 pl fruit$^{-1}$ h$^{-1}$) recorded from control plants (Fig. 4A). This production rate was not significantly different from untreated control fruit at DS3 (Fig. 3A).

In contrast to the flowers and fruit at DS3, fruits at DS4 and DS6/24 h+ responded differently to STS exposure. Control fruit at DS4 that had been injected with water showed a normal pattern of $\text{C}_2\text{H}_4$ production (Fig. 4C; square and triangle symbols); the expanded pale-green fruit showed a diurnal production of normal amplitudes and duration. The injection of STS into fruit at DS4 caused a reduction in $\text{C}_2\text{H}_4$ production and a loss of the diurnal periodicity. The rate of $\text{C}_2\text{H}_4$ production by water-treated DS4 fruit was approximately 270 pl fruit$^{-1}$ h$^{-1}$ (Fig. 4C; diamond symbols). Control fruit at DS6/24 h+ that had been injected with water also showed a level of $\text{C}_2\text{H}_4$ production that was similar.
to non-injected fruit (and therefore a graph is not shown). Fruit at DS6/24 h+ exhibited reduced C₄H₄ production after STS treatment. Typically, water-injected control fruit evolved significantly more (ANOVA; P < 0.001; approximately three-fold) C₄H₄ than the STS-treated fruit: for example, 165 ± 0.2 pl fruit berry⁻¹ h⁻¹ (n = 3) and 60 ± 0.1 pl fruit berry⁻¹ h⁻¹ (n = 3), respectively.

**Immunolocalization of ACO**

Our laboratory studies supported previous results (e.g. Tian et al. 2000) that found the exogenous application of aminoethoxyvinyl glycine (AVG), 1-MCP or STS to have no arresting effect on the ripening process. Therefore, and because achene-derived auxin plays a pivotal role in strawberry fruit ripening (Medina-Escobar et al. 1997), the relative potential contribution of the flesh and achene to produce C₄H₄ was therefore first investigated by using Western analysis of de-achedened fruit-flesh and achene-only proteins (Fig. 5) using an IgG raised against the protein product of TR-ACO2, a member of the ACO gene family from white clover (Trifolium repens L.; Hunter et al. 1999). The only immunoreactive antigen at the expected predicted relative molecular mass (Mᵣ, 40 kDa) for ACO was detected in the achene-only sample (Fig. 5; lane 3). In this extract, a protein with an Mᵣ of approximately 30 kDa is also recognized. In the plant species that have been analysed to date, ACOs are characterized as monomeric with an Mᵣ that range from approximately 35 to 40 kDa (Gong and McManus 2000), although a 27.5-kDa peptide has been reported in papaya fruit (Dunkley and Golden 1998). At this stage of our investigation, it is not clear what relationship exists between the 30 and 40 kDa proteins, and further characterization is needed to establish a definitive relationship.

Further localization studies using the same antibody in SE-IgL of fruit flesh and achenes of fruit developing from DS3–4 (i.e. during expansion) and DS6/24 h+ showed that immunoreactive antigens could be visualized strongly in embryo tissue, within the seeds of expanding (DS4; Fig. 6B) and ripe fruit (DS6/24 h+ Fig. 6C) only. Fruit flesh labelled only weakly in all DSs examined (data not shown). This relative difference in SE-IgL intensity between the flesh and seeds may explain the absence of an immunoreactive band in Western blots of ‘epidermal peel plus achene’ extracts, particularly because ripe strawberry fruit extracts have a natural capacity to bind high levels of protein (Souleyre et al. 2004). Despite this, our findings correlate with the physiology measurements: that significant levels of C₄H₄ are produced by expanding fruit and fruit that has been red ripe for at least 24 h. Furthermore, these data suggested that the ACO antigen appears concentrated within the seed, indicating that significant amounts of C₄H₄ may be derived from this tissue.

**C₄H₄ production from dissected fruit parts**

Assessment of the relative rates of C₄H₄ production from dissected fruit parts showed that the damage to the fruit achened epidermis that simulated the disruption to the epidermis during achene removal significantly altered C₄H₄ production in fruit at DS6/24 h+ and DS4. Red-fruits (DS6/24 h+) epidermis peels showed a 50 ± 7% increase in the amount of C₄H₄ produced, and DS4 fruit peel showed a 21 ± 4% reduction. It was therefore possible to correct the values obtained from de-achedened epidermal peels to compensate for the effects of damage. On this basis, C₄H₄ could not be detected from dissected fruit epidermis or the achenes at late DS4/DS5. In contrast, the levels of C₄H₄ evolved from epidermal peels minus achenes were approximately three-fold higher (280 ± 90 pl h⁻¹ g⁻¹ FW; Table 1) at DS4 when compared with DS6/24 h+ (80 ± 20 pl h⁻¹ g⁻¹ FW; Table 1). The C₄H₄ evolved from achenes did not vary significantly between DS4 and DS6/24 h+ (808 ± 90 and 790 ± 180 pl h⁻¹ g⁻¹ FW, respectively; Table 1). On a tissue FW basis, achenes evolved approximately four-fold to 10-fold more C₄H₄ per gram compared with fruit epidermal peels of DS4 and DS6/24 h+, respectively (Table 1). Estimates of C₄H₄ production in absolute terms show approximately four-fold higher production rates for the epidermis of fruit at DS4 (34 ± 7 pl h⁻¹) than at DS6/24 h+ (9.5 ± 1.6 pl h⁻¹; Table 1). However, by DS6/24 h+, absolute production rates for fruit epidermal peels and achenes do not differ
significantly (9.2 ± 0.6 and 9.5 ± 1.6 pl h⁻¹, respectively; Table 1). It should also be noted that experiments, which examine the impact of STS application on dissected achene and fruit epidermal peel, remain to be carried out. Such analyses would discern whether the true source of the switch in C₂H₄ self-regulation from negative to positive resides in either the epidermis or seed tissue or both.

**Discussion**

The results presented here conflict with notions that stereotype strawberry fruit growth and ripening as a non-climacteric fruit. We provide unequivocal evidence that C₂H₄ production by developing and ripening strawberry flowers and fruits maturing from DS3 to DS6/24 h+ occurs in a definite and highly reproducible manner that correlates with distinct DSs (Figs 2 and 3). C₂H₄ production during flower opening and senescence and by fruit developing from DS3 to DS4 is characterized by cyclic diurnal production (Figs 2 and 3) that correlates with the growth and expansion of the fruit and chlorophyll loss. We also present clear evidence that C₂H₄ production by red-ripe fruit is characterized by a non-diurnal cyclic accumulation that coincides with a respiratory climacteric (Fig. 3B). Our in planta findings also contradict reports, gathered from detached and dissected strawberry fruit, that suggested strawberry fruit C₂H₄ production to be under negative-feedback control throughout ripening (Mordy et al. 2000). Our data show that the pattern of C₂H₄ production by strawberry fruit is similar to that exemplified by tomato, where C₂H₄ production is self-regulating via negative-feedback processes until the mature expanded white or pale-green stage, when regulation switches to positive control (Fig. 4). Our approach, to administer STS by injection, took into account the fact that there is no physiological effect on the developmental progress of ripening strawberry when STS, 1-MCP or AVG is administered exogenously and that the levels of C₂H₄ produced from within the seeds are high (as compared with the epidermal output) and may be particularly significant in ripe fruit (Table 1). The physiological response of STS injected into the vascular traces of strawberry flowers and fruits at DS3 suggests that C₂H₄ production is self-regulating via a negative-feedback control mechanism (Fig. 4A, B). In contrast, STS caused C₂H₄ to be evolved by positive feedback by fruit at DS4 (Fig. 4C) and DS6/24 h+. In addition, data quantifying the amount of C₂H₄ generated due to tissue damage (removal of achenes) revealed a significant reduction (approximately 20%) in C₂H₄ production in expanded white-fruit peel (DS4), whereas red-fruit peel showed increased (approximately 50%) C₂H₄ production. This also highlights the existence of a switch in C₂H₄ self-regulation from negative- to positive-feedback control (respectively) as fruit mature from DS3/4 to DS6 + 48 h.

**Table 1.** Ethylene production rates (corrected for response to damage during dissection) by the strawberry fruit samples: epidermal peel minus achenes and achenes only from fruit at developmental stage 4 (DS4) and 6. Data are expressed in absolute (pl h⁻¹) and relative (pl h⁻¹ g⁻¹ FW) terms. *Samples that are significantly greater (ANOVA; P < 0.001). NS, non-significantly different samples.

<table>
<thead>
<tr>
<th>Ethylene production rate</th>
<th>Standardized for fresh weight (pl h⁻¹ g⁻¹ FW)</th>
<th>Absolute production (pl h⁻¹)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Epidermal peel minus achenes</td>
<td>Achenes only</td>
</tr>
<tr>
<td>DS4</td>
<td>280 ± 90</td>
<td>810 ± 90⁺</td>
</tr>
<tr>
<td>DS6/24 h+</td>
<td>80 ± 20</td>
<td>790 ± 180⁺</td>
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![Fig. 6. Silver-enhanced immunogold-labelled light micrographs of strawberry seeds. (A) Developmental stage 4 (DS4) seed negative-control section probed with (pre-immune) normal rabbit serum. (B) DS4 seed and (C) DS6 seed labelled with anti-1-aminocyclopropane-1-carboxylic acid oxidase IgG. e, embryo cells; s, seed coat. Scale bars = 100 μm (A, B) and 50 μm (C).](image-url)
The switch in diurnal growth-associated \( \text{C}_2\text{H}_4 \) production to one of continuous \( \text{C}_2\text{H}_4 \) production (Fig. 3A) after a 24-h lag phase may reflect a switch from processes governing fruit development and ripening to those that encourage fruit senescence-mediated processes that include fruit softening and microorganism (\textit{Botrytis}; grey mould) mediated pathogenesis – all of which may be encouraged by the elevated levels of \( \text{C}_2\text{H}_4 \) (Kepczynska 1989, Tian et al. 2000, Castillejo et al. 2004, respectively) and to which seed derived \( \text{C}_2\text{H}_4 \) contributes significantly.

The results presented here also highlight that the term ‘climacteric’ may be considered as scientifically ‘under discussion’, as it can no longer be applied consistently to account for the underlying biological complexity of (at least) strawberry fruit ripening. Our data do clearly demonstrate that, by classically physiological definition, strawberry is justifiably a climacteric fruit, because a respiratory increase concomitant with a \( \text{C}_2\text{H}_4 \) increase does occur in ripening strawberry fruit (Fig. 3B). However, we acknowledge that our results are correlative and do not prove a causal role for \( \text{C}_2\text{H}_4 \) in strawberry fruit ripening. Despite this, we also highlight that stereotyping fruit that produce very low levels of \( \text{C}_2\text{H}_4 \) as ‘non-climacteric’ based on whether \( \text{C}_2\text{H}_4 \) appears to be a prerequisite for fruit ripening may be misleading; this approach fails to consider the underlying biology that will determine the absolute quantity of \( \text{C}_2\text{H}_4 \) (or efficacy \( \text{C}_2\text{H}_4 \) interfering chemicals) to which a physiological response can/cannot occur. We suggest that the \( \text{C}_2\text{H}_4 \)-sensing mechanism in climacteric fruit may be so sensitive as to preclude the appearance of a phenotypic effect ex post facto. That is, the efficacy with which \( \text{C}_2\text{H}_4 \) inhibitors (such as STS or 1-MCP) can saturate all the available \( \text{C}_2\text{H}_4 \) receptors in strawberry tissues and arrest the \( \text{C}_2\text{H}_4 \) response might be low, whereas the biological efficacy of \( \text{C}_2\text{H}_4 \) receptor may be high. In addition, we have demonstrated that \( \text{C}_2\text{H}_4 \) production from strawberry seeds is significant, and this tissue may be considered particularly inaccessible to standard inhibitor applications, compared with receptors of the flesh. This possibility is supported by recently reported molecular data (Trainotti et al. 2005) demonstrating that \textit{Fa ACOI} gene expression correlates with \( \text{C}_2\text{H}_4 \) increases in tandem with the switch in \( \text{C}_2\text{H}_4 \) regulation from negative- to positive-feedback control, postulating a possible role for this gene/mechanism in signalling the progression of the strawberry ripening process. Furthermore, concomitant with this is the increased synthesis of type II receptors (\textit{FaETR2}), whereby low levels of \( \text{C}_2\text{H}_4 \) may be sufficient to promote ripening-related responses (Trainotti et al. 2005). This opportunity could be exploited further using readily available microarray tools (Aharoni et al. 2000), and the genetic studies to dissect a direct analyses on the role of \( \text{C}_2\text{H}_4 \) in the ripening process in strawberry should be targeted towards \textit{ACO} and \textit{ACC-synthase} and \( \text{C}_2\text{H}_4 \) receptors expressed in the fruit flesh or (arguably), more importantly, the seed, because our results from dissected fruit showed clearly that the levels of \( \text{C}_2\text{H}_4 \) produced by the ‘true fruits’ achenes are significant, especially for ripe fruit (Table 1). The possibility that there is a temporal separation of \( \text{C}_2\text{H}_4 \) production initiation between achene and flesh tissue requires investigation. That is, in the same way that declines in achene-derived auxin promotes ripening (Reddy and Poovaiah 1990, Reddy et al. 1990), so may achieve-\( \text{C}_2\text{H}_4 \). This significance of this if proven may explain the inefficiency of attempts to arrest strawberry fruit ripening using \( \text{C}_2\text{H}_4 \) receptor inhibitors.

Ultimately, the causal role for \( \text{C}_2\text{H}_4 \) in post-harvest processes must ultimately be assessed in transgenic strawberry plants with blocked \( \text{C}_2\text{H}_4 \) production and sensing, as has been demonstrated for tomato (Gray et al. 1993) and melon (Ayub et al. 1996). Samples selected for their physiological state (defined in this instance as \( \text{C}_2\text{H}_4 \) production) will facilitate this full and accurate molecular dissection of \( \text{C}_2\text{H}_4 \)-related strawberry fruit ripening. For example, we have shown that fruit at the visual phenotype DS4 may or may not have stopped evolving \( \text{C}_2\text{H}_4 \) in a diurnal pattern cyclically and may or may not have entered the phase where \( \text{C}_2\text{H}_4 \) production in under positive regulation. In this way, fruit at DS4 could have three possible \( \text{C}_2\text{H}_4 \)-related physiological phenotypes. The LPD (http://www.sensor-sense.nl) will therefore allow specific physiological stages to be identified, based on the pattern and/or quantity of \( \text{C}_2\text{H}_4 \) that is produced.

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