Ethylene Biosynthesis during Peach Fruit Development

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Abstract. Ethylene evolution and ACC levels were determined throughout the growth and development of peach fruit (Prunus persica L. Batsch cv. Redhaven). In the four stages of growth (I, II, III, IV), as indicated by weekly monitoring of fresh (FW) and dry (DW) weight accumulation, ethylene biosynthesis in whole fruit decreased during FWI and remained almost undetectable during FWII and FWIII. In pericarp disks, ethylene evolution followed the same trend, although a peak at 78 days after full bloom and a slight increase before the onset of the climacteric were observed. The high rates of ethylene evolution were associated with a concurrent increase in ACC content. Enhancement of ACC synthase and ethylene-forming enzyme (EFE) activities was responsible for the peak of ethylene evolution detected before the beginning of FWIII and DWIII. At the climacteric, which occurred at the FWIII-FWIV transition, sequential events were observed in different fruit tissues. An increase of ethylene production in the mesocarp preceded the onset of the climacteric rise in whole fruit. The high amount of ethylene detected during the climacteric appeared to be related to increased EFE activity in the epicarp. Chemical name used: 1-aminocyclopropane-1-carboxylic acid (ACC).

In climacteric fruit, ethylene production rises dramatically at the initiation of ripening, a stage of development in which the hormone is believed to play a crucial role. The hypothesis of ethylene involvement in the regulation of genes controlling ripening has been successfully demonstrated in several fruits (Christoffersen et al., 1989; Giovannoni et al., 1989; Grierson et al., 1985, 1989; Lincoln and Fischer, 1988; Lincoln et al., 1987; Tucker et al., 1985).

Physiological and biochemical processes, other than ripening, that characterize fruit development may be regulated by hormones and, specifically, by ethylene. The early work of Chalmers and van den Ende (1975) and Jerie and Chalmers (1976) observed a concurrent increase in ethylene evolution and dry weight accumulation in long-season peaches during the late stage of rapid dry weight gain (DWII).

Since Adams and Yang (1979) established that ACC is the immediate precursor of ethylene, various studies have been published on the kinetics of ethylene evolution, ACC concentration, ACC synthase, and EFE activity in fruit tissues, particularly in relation to the climacteric peak. Miller et al. (1988) studied ethylene evolution and ACC content of peach fruit throughout development.

The research reported in this paper studies the relationships of ACC synthase, EFE activity, and ACC conjugation to the ethylene evolution pattern throughout peach fruit growth and development. Attention was focused on the onset of the climacteric, when the highest rates of macromolecular synthesis occur (Richmond and Biale, 1966, 1967; Romani, 1978) and when System I triggers the formation of System II ethylene (McMurchie et al., 1972). System I is responsible for basal ethylene production in fruits before the onset of ripening, and System II accounts for the autocatalytic rise of ethylene accompanying ripening.

Materials and Methods

Plant material. ‘Redhaven’ peaches were harvested once a week in 1987, 1988, and 1989 at the Univ. of Padova Research Farm, Italy, beginning =30 days after full bloom (AFB). Fresh and dry weight (48 h at 70C) determinations were carried out weekly on average-sized fruits, as determined by measuring the diameter of 200 fruits every 2 days from fruit set until ripening.

Ethylene determination. Whole-fruit ethylene production was determined by enclosing fruits in jars (0.2 to 1 liter) sealed with a rubber cap and kept in the light at 25C. After 4 h, a 1-ml air sample was withdrawn from each jar for ethylene measurement. Disks of pericarp (including all fruit parts before endocarp lignification), mesocarp, and epicarp were separately enclosed in test tubes (21 ml) with 0.5 ml of water to avoid dehydration. Since the ethylene production rate of the disks did not change significantly within the 2nd and 7th h of incubation (data not shown), the ethylene measurements were made within that time.

A gas chromatography (Perkin-Elmer F17; Norwalk, Conn.) equipped with a flame ionization detector and an alunina column was used for quantifying ethylene concentrations.

Determination of ACC and 1-(malonylamino) cyclopropane-1-carboxylic acid (M-ACC). Pericarp, mesocarp, and epicarp tissues were incubated in 5 ml of 80% ethanol at 70C for 30 min and then homogenized. After centrifugation at 10,000x g for 15 min, the supernatant was passed through columns of polyvinylpirrolidone (PVP) (3 ml). Authentic ACC was eluted from the column in the first and second 1-m1 fractions; therefore, the columns were eluted with two fractions of 1 ml of water, and ACC was determined according to Lizada and Yang (1979). The efficiency of the conversion of ACC to ethylene was routinely 80% to 85%. Conjugated ACC was measured after hydrolysis, as described by Hoffman et al. (1982).

Extraction and assay of ACC synthase. Mesocarp and epicarp tissues (3 g fresh weight) were frozen in liquid nitrogen and ground to a powder. Three milliliters of extraction buffer [Hepes-
KOH, 100 mM, pH 8.5; dithiothreitol (DTT), 4 mM; pyridoxal phosphate, 2 µM; PVP, 0.2% (w/v) were added to the powder. The extract was centrifuged for 20 min at 14,000× g, and the supernatant was dialyzed overnight against a solution containing 2 mM Hepes-KOH (pH 8.5), 0.1 mM DTT, and 1 µM pyridoxal phosphate. All these steps were carried out at 4°C. ACC synthase was determined by incubating at 30°C for 1 h in the dark in a solution containing 0.4 ml of the crude extract, 0.1 ml of 0.3 M Hepes-KOH (pH 8.5), and 0.1 ml of 0.3 mM s-adenosylmethionine (SAM). ACC content was then determined as previously described.

Protein concentration was determined by the method of Bradford (1976).

Assay of EFE. EFE was assayed in vivo by incubating pericarp, mesocarp, and epicarp disks in 30 µM cycloheximide (CHI) for 30 min and then for 3 h in 5 mM ACC solution. Tissues were then transferred to test tubes sealed with rubber caps, and ethylene was determined after 1 h.

Whole fruit were treated 90 and 103 days AFB with air or ethylene (10 µl·liter⁻¹) for 15 h, and disks of mesocarp were then assayed for ethylene production and EFE activity as described above.

To gain a better insight into the events characterizing the onset of the climacteric during late FWIII and early FWIV, all the determinations were repeated on single fruits. Each fruit was weighed and its physiological stage in relation to the climacteric was assessed by measuring ethylene evolution.

The experiments performed in all 3 years gave similar results. Excluding the data in Table 1, which were collected in 1989, the results reported are from 1988.

With the exception of the ACC synthase activity experiment (Fig. 4), values are means of at least three replicates.

Results

Growth analysis. The fruit FW and DW accumulation followed the classical double-sigmoid curve. In accordance with Chalmers and van den Ende (1975), the growth patterns were analyzed by using the first derivative and eight stages—FWI, FWII, FWIII, FWIV, DWI, DWII, DWIII, and DWIV—were identified. Transitions between stages occurred at 55, 84, and 103 days AFB and 77, 84, and 97 days AFB for FW and DW, respectively (Fig. 1).

Ethylene production. The whole-fruit ethylene evolution at 33 days AFB was 0.67 nl/g FW per hour (Fig. 2). Later, it slowly decreased and remained almost undetectable through FWII and FWIII. The climacteric rise started after 97 days AFB.

The basal pericarp ethylene evolution was higher than that of the whole fruits (Fig. 2). This might reflect the effect of wounding, which affected ethylene biosynthesis within 1 h. Nevertheless, the ethylene production in whole fruits and in pericarp disks displayed the same trend, showing steady low values during the growth cycle and an increase in relation to the climacteric. Unlike for whole fruit, in the pericarp disks, a small peak (3.85 nl·g⁻¹·h⁻¹) appeared 78 days AFB and a slight increase 97 days AFB, just before the onset of the climacteric rise (Fig. 2).

ACC and M-ACC content and ACC synthase activity. ACC content in the pericarp was 2.25 nmol/g FW at 33 days AFB (Fig. 3), then declined and remained low during FWI and part of FWII. Later it increased, peaking (2.67 nmol/g FW) at 78 days AFB. A second rise of ACC content was concurrent with the onset of ethylene production in whole fruit.

Enhanced activity of ACC synthase was found in the mesocarp during late FWII (Fig. 4). No increase of ACC synthase activity was observed during the ethylene climacteric rise. The activity of this enzyme remained undetectable throughout the fruit growth cycle in the epicarp tissue.

The pericarp M-ACC content paralleled the ACC level (Fig. 5). A first peak was observed during FWII and a second one just before the climacteric.

EFE activity. EFE activity throughout FWII, FWIII, and FWIV in the pericarp (Fig. 6), mesocarp, and epicarp (data not shown) disks showed the same pattern, peaking at 78 days AFB, declining during FWIII, and increasing again at the climacteric.

Experiments carried out with CHI during three stages of fruit development (Table 1) pointed out that epicarp and mesocarp
respond differently to wounding. In the climacteric stage (120 days AFB), ethylene evolution by epicarp disks pretreated with CHI was higher than in mesocarp.

In preclimacteric fruit, the mesocarp ethylene evolution reached the highest value 103 days AFB, e.g., before the onset of the climacteric rise in the whole fruit (Table 2). A clear increase in ACC levels was observed in mesocarp at the preclimacteric stage. The enhanced rate of ethylene production observed in mesocarp disks at 103 days AFB apparently was a consequence of the increased EFE activity. It is noteworthy that at 120 days AFB, when the whole fruit clearly was in the climacteric stage, ethylene evolution from mesocarp had not risen further, although ACC and EFE activity were high. Considering the ACC concentration and ethylene evolution of the mesocarp, it appeared that the conversion efficiency of the precursor to ethylene was very low. It may be that a large part of the measured ACC is sequestered and not available for the EFE catalytic action. In the epicarp, the basal ethylene production was higher than in the mesocarp due to the effect of wounding, and the evolution pattern paralleled the climacteric rise in whole fruit. The epicarp ACC content was lower than in the mesocarp, even during the

Table 1. Effect of CHI on ethylene evolution in mesocarp and epicarp disks of peach fruit at three stages of ripening, expressed as AFB. Effect of wounding was evaluated by incubating disks in CHI (30 μM for 30 min) immediately after excision. Ethylene was measured after 3 h of incubation.

| Days AFB | Mesocarp | Epicarp |
|----------|----------|---------|
|          | − CHI | + CHI | − CHI | + CHI |
| 93       | 0.19 ± 0.05 | ND | 0.92 ± 0.16 | ND |
| 100      | 0.23 ± 0.04 | ND | 2.89 ± 1.84 | 0.09 ± 0.02 |
| 120      | 2.07   | 0.16 ± 0.05 | 11.24 ± 2.57 | 0.53 ± 0.13 |

ND = not detectable.
climacteric, while the EFE activity was consistently higher. A dramatic rise of the same enzymatic activity occurred 120 days AFB, e.g., concurrently with the highest value of ethylene production in whole fruit.

Exogenous ethylene (10 µl·liter⁻¹, applied to preclimacteric (90 days AFB) and climacteric (103 days AFB) fruits did not affect mesocarp ethylene evolution, while EFE activity was stimulated only when ethylene was applied during the climacteric (Fig. 7).

### Discussion

Peach fruit growth is characterized by cell division during early FWI and, later, by cell enlargement (Vizzotto et al., 1989). Unlike for the conditions of Chalmers and van den Ende (1975), under our climate, FW and DW stages were not completely displaced in time. In fact, although FWI and DWI lasted 55 and 77-days, respectively, FWIII and DWIII both began 84 days AFB.

A decrease in ethylene production has been reported to be responsible for the FWI–FWII transition. An application of exogenous ethylene reduced the lag phase of growth (Marei and Crane, 1971; Zeroni et al., 1972). In our study, a decrease in ethylene evolution was detected in both whole fruit and pericarp during FWI, confirming what has been found by others (Jerie and Chalmers, 1976; Looney et al., 1974; Miller et al., 1987, 1988). Whether or not the high amount of ethylene detected at the beginning of FWI plays a role in growth remains to be established. The relatively high ACC content measured at 33 days AFB might indicate an activation of the biosynthetic pathway and could be related to the stimulating effects on ACC synthase activity induced by the high 1-H-indole-3-acetic acid (MA) concentration detected in early FWI (Miller et al., 1987; Vizzotto et al., 1989).

The increased ethylene evolution observed 78 days and 97 days AFB in pericarp tissue might reflect physiological changes occurring within the fruit. The first peak appears associated with an activation or a de novo synthesis of ACC synthase (Fig. 4) and of EFE (Fig. 6) in the mesocarp. Attempts to determine the ACC synthase activity in the epicarp were unsuccessful throughout the fruit growth cycle. Lack of success might be related to low activity of the enzyme, since the ACC content in the same tissue was lower than in mesocarp, both in preclimacteric and climacteric fruit (Table 2). The absence of ACC synthase activity in the mesocarp during the climacteric may support the hypothesis of different forms of ACC synthase. The form appearing during the climacteric could be extremely unstable or specifically inhibited or inactivated by the extraction procedure. Alternatively, an endogenous inhibitor that appears during the climacteric may be hypothesized. Imaseki et al. (1989) suggested that in squash mesocarp at least two genes code for ACC synthase, one activated by wounding and the other by auxin. The two isoforms are immunochemically different. The same authors suggested that ethylene biosynthesis may be physiologically classified into three types: auxin-regulated, stress-regulated, and ripening-associated biosynthesis. Since the ethylene (and ACC) peak observed 78 days AFB appeared concurrently with an increase in mesocarp IAA concentration (Vizzotto et al., 1989), we suggest that, in peach fruit, the modulation of ethylene biosynthesis may be regulated by auxin at the beginning of FWI and at the FWII-FWIII transition and by the ripening-associated events during the climacteric.

It is generally accepted that EFE is constitutive in vegetative tissue but not in preclimacteric fruit (Yang and Hoffman, 1984).
This fact is confirmed by our findings in mesocarp tissue. Peculiar to peach fruit is the high EFE activity of the epicarp even in preclimacteric stages (Table 2) and the small but significant increase of EFE activity in the pericarp, which occurs, together with a rise in ACC content, just before the beginning of FWIII (Fig. 6). The concomitant increase of ethylene production might be related to a direct action of the hormone on cell enlargement, a process which characterizes FWIII. Inhibitors of ethylene biosynthesis or action have been shown to be active in reducing or limiting cell enlargement in wheat coleoptiles (Ramina and Tonutti, 1984). Although no correlation was found between ethylene biosynthesis and DWI and DWII, a possible involvement of the hormone in some other process characterizing peach fruit growth after pit hardening cannot be ruled out.

Malonylation of ACC has a role in regulating the amount of ACC available for ethylene biosynthesis. In avocado fruit, malonylation of ACC regulates ethylene production during the preclimacteric but not the climacteric stage (Sitrit et al., 1986). Mansour et al. (1986) suggested that, in preclimacteric apples, the high level of M-ACC might be involved in the regulation of ethylene biosynthesis. Although the M-ACC (and ACC) levels detected in peach mesocarp are much lower than those found in avocado and apple, the increase in M-ACC concentration detected during FWII and FWIII (Fig. 5) is consistent with a regulatory role of the malonylation process, especially when ethylene biosynthesis is low (78 days AFB). At the climacteric stage, M-ACC content first (97 days AFB) increases, then declines concurrently with a rise in EFE activity. This sequence may suggest a role of ACC in regulating the onset of the increase in ethylene evolution, which is concomitant with the climacteric during fruit ripening.

In whole fruit, the climacteric is concurrent with the FWIII-FWIV transition. Although the high ethylene production seems to be mainly in the epicarp (Table 2), the onset of the climacteric at 109 days AFB in whole fruit, when ethylene evolved at 1.83 nl·g⁻¹·h⁻¹ (Table 2), was preceded by a 6-fold increase in ethylene evolution (2.45 nl·g⁻¹·h⁻¹) by the mesocarp 103 days AFB. In the same tissue, the ACC content rises before the EFE activity, whereas in the epicarp, the most important event is the dramatic increase of EFE activity. Basal ethylene production of the epicarp is consistently higher than that of the mesocarp even in the preclimacteric stage, despite the low ACC content. This relationship may reflect a peculiarity of the peach epicarp, which, being extremely sensitive to wounding (Table 1), could be adapted to react to physical injuries by increasing ethylene biosynthesis.

The observed difference between epicarp and mesocarp and the effect of exogenous ethylene on ethylene production and EFE activity of mesocarp (Fig. 7) may indicate that: 1) in mesocarp tissues, a close correlation exists between ACC content and ethylene production; 2) the increase in ethylene evolution in both the whole fruit and epicarp is preceded by an increase in ethylene evolution in the mesocarp; 3) the dramatic increase in EFE activity in the epicarp during the climacteric might be responsible for the relatively high amount of ethylene produced by whole fruit; and 4) the enhanced sensitivity of mesocarp tissue to exogenous ethylene slightly affects ethylene production but dramatically increases EFE activity. Although exogenous ethylene has been shown to stimulate ACC synthase activity (Bufler, 1984), its main effect might be related to the conversion of ACC to ethylene (Bufler, 1986; Liu et al., 1985).

Our data confirm that, in relation to the ethylene biosynthetic pathway, a gradient exists within the fruit, the epicarp being the most efficient tissue in converting ACC to ethylene. Furthermore, in relation to the System I and System II theory (McMurvie et al., 1972), the crucial event leading to the climacteric could be represented by the increase of EFE activity occurring in the mesocarp. Whether this is a consequence of a slight stimulation of ethylene biosynthesis or an enhanced sensitivity to the hormone remains unclear. This step may represent the key to the transition from System I to System II responsible for the autocatalytic ethylene production.

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