Some Biochemical and Ultrastructural Aspects of Peach Fruit Development

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Additional index words. free IAA, peroxidases, IAAoxidases, growth stages, secretory system, mucilages

Abstract. 'Redhaven' peach [*Prunus persica* (L.) Batsch.] fruit growth, expressed as cheek diameter, displayed a double-sigmoid pattern in which four stages were defined (SI, SII, SIII, SIV). Free IAA concentration, as determined by polyclonal antibodies (PcAb) enzyme-linked immunosorbent assay (ELISA), paralleled fruit growth rate, peaking at 30 and 85 days after full bloom (AFB), concurrently with the exponential phases of growth. The highest peroxidase (EC 1.11.1.7) (POD) and IAA oxidase (IAAox) activities occurred during endocarp lignification. The main structural events described were mesocarp cell division within the first 2 weeks AFB and, later, cell enlargement, modifications of the epicarp cells, lignification of the endocarp, differentiation of the chloroplasts, and changes in their starch content. Chemical name used: indole-3-acetic acid (IAA).

The key role of IAA in fruit development is generally accepted, although in pome and stone fruits exogenous application of auxins failed to promote growth (Coombe, 1976; Crane, 1969; Wertheim, 1971). Nevertheless, Nitsch (1954) showed that auxin was related to cell enlargement in strawberry (*Fragaria × ananassa* Duch.), and the opinion persists that IAA, either directly or indirectly, controls fruit growth and development. In peach, the IAA content in pericarp and seed has been related to diameter increase and fresh weight gain (Miller et al., 1987; Quesada et al., 1990; Sanchez-Rolan et al., 1990; Valpuesta et al., 1989; Vizzotto et al., 1989).

In this paper we examine the relationship between auxin and fruit growth by relating changes in IAA concentration, IAAox, and POD activities in the pericarp/mesocarp to the stages of fruit development. In addition, peach fruit ultrastructure was characterized to gain insight into the changes occurring during fruit development.

Materials and Methods

Plant material. Developing fruits of 'Redhaven' were harvested in 1989 at 3- to 4-day intervals, beginning 7 days AFB until ripening, from trees grown at the experimental farm of the Univ. of Padova (Legnaro, Padova, Italy). On each sampling date, observations and analyses were carried out on fruits whose size conformed to the average determined by measuring the cheek diameter of 200 fruits. Fruit growth analysis was carried out by constructing a cumulative growth curve. Stage transitions were marked using the rate of increase in diameter (Chalmers and van den Ende, 1975). According to this method, four stages (SI, SII, SIII, and SIV) were defined.

Biochemical analysis. Up to 60 days AFB, all the biochemical determinations were carried out on the pericarp. Thereafter, epicarp and endocarp were dissected from the mesocarp, and the mesocarp was used for analysis.

a) Extraction and purification of IAA. Extraction and purifi-

cation of free IAA were performed according to Thompson et al. (1981), with modifications. Fresh tissue (0.5 to 1 g) was ground in a cold mortar and pestle, then extracted with 5 ml of methanol : 100 mM ammonium acetate (NH₄OAc) (80:20 v/v) + 2,6-tert-butyl-4-methylphenol (BHT) (45.4 μM) at 4°C. The homogenate was transferred to a centrifuge tube and maintained at 4°C for 30 min, then centrifuged at 16,000 × g for 20 min. The pellet was resuspended in 5 ml of cold distilled water and centrifuged at 10,700 × g for 10 min. Both supernatants were pooled and 1-¹⁴C-labeled IAA (24 μM, 2.11 GBq·mmol⁻¹) was added to each sample as internal standard. The supernatant was loaded onto a polyvinylpyrrolidone (PVPP) column and the eluate was processed through a second DEAE-Sephadex A 25 column (acetate form). The two columns were washed with 30 ml of 10 mM NH₄OAc and IAA was eluted from the DEAE-Sephadex column with acetic acid (1 M) + BHT (45.4 μM).

The radioactive fractions were loaded into a C-18 Sep-pak (Waters, Milford, Mass.) disposable cartridge that had been primed with methanol + BHT (45.4 μM), washed exhaustively with distilled H₂O and 10 ml of acetic acid (5 mM), then purged with N₂ before use. The column was then washed with distilled water to remove the acetic acid, purged with N₂, and IAA eluted with 5 ml of methanol (HPLC grade) + BHT 45.4 μM in a Sovirel SVL22 vial.

The methanol solution was reduced to the aqueous phase in a rotary evaporator at 35°C and dried under a stream of N₂. The dried sample was redissolved in 6 ml of 1 MeOH : 1 diethylether solution (v/v), methylated with an excess of ethereal diazomethane according to Walker et al. (1982), and then dried with N₂. All the samples were stored at -20°C until the ELISA immunoassay was performed. All the glass- and plasticware was silylated to minimize sample losses.

b) IAA ELISA immunoassay. Anti-IAA gamma immuno-globulins (IgG), as crude serum, were purified and concentrated according to Clark and Adams (1977). IAA-alkaline phosphatase (IAA-aP; EC 3.1.3.30) tracer was synthesized following procedures of Weiler (1986). The IAA standard was prepared according to Sagee et al. (1986). Assay optimization of plant material was by standard ELISA protocol (Sagee et al., 1986).

Three extracts were prepared from each sample and the measurements for each extract were repeated three times. Quantitation of the results was by internal standardization (Crozier et

Received for publication 10 July 1991. Accepted for publication 18 Feb. 1992. Authorized for publication as paper no. 254 of the Scientific Journal Series of the Institute of Pomology, Univ. of Padova, Italy. We acknowledge R. Goren from the Hebrew Univ. of Israel for providing the anti-IAA polyclonal antibodies. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked advertisement solely to indicate this fact.

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This paper is dedicated to the memory of Piero.

Abbreviations: AFB, after full bloom; FW, fresh weight; IAAox, IAA oxidase; PAS, periodic acid-Schiff's reagent; PcAb-ELISA, polyclonal antibodies enzyme-linked immunosorbent assay; PVPP, polyvinylpyrrolidone.

al., 1986; Pengelly, 1986; Reeve and Crozier, 1980; Weiler et al., 1986).

c) *Enzyme extraction.* Fruits were harvested every 4 days, and tissue was dissected and stored at -20°C for determination of IAAox and POD activity.

Two- to 4-g fresh weight (FW) samples of pericarp/mesocarp tissues were homogenized in phosphate buffer (200 mM, pH 7.0) + PVPP ($1\text{ g}\cdot\text{g}^{-1}\text{ FW}$). The homogenate was filtered with a nylon gauge and centrifuged at $25,000\times g$ for 15 min. The supernatant was used for the evaluation of enzyme activity. IAAox activity was determined according to Rubery (1972) and Richard and Job (1974) and POD activity according to Chance and Maehly (1955).

Light and electron microscopy. Samples of tissue taken from various pericarp regions were fixed overnight in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 6.9), postfixed 2 h in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 6.9), and dehydrated in a gradient series of ethyl alcohol and propylene oxide. Tissues were stained with uranyl acetate while dehydrating in 75% alcohol. Tissues were embedded in an Epon-Araldite mixture. Ultrathin sections (500–600 Å) cut with an ultramicrotome (Ultracut, Reichert-Jung, Vienna) were post-stained with lead citrate and examined with a transmission electron microscope (HS9 Hitachi, Tokyo) operating at 75 kV. Thin sections ($1\text{ }\mu\text{m}$) obtained by the same ultramicrotome were stained with 1% toluidine blue : 1% tetraborate (v/v) and observed and photographed under a Leitz (Wetzlar, Germany) Ortolux light microscope.

Cytochemical tests. Thin sections cut from resin-embedded samples were treated with periodic acid-Schiff's reagent (PAS) to stain polysaccharides (Jensen, 1962). Sections from fresh samples of pericarp tissue were cut with a Criocut E (Reichert-Jung) and submitted to the Hoepfner Vorsatz test (Reeve, 1951) to assay for phenolic compounds.

Results

Growth analysis. Growth in diameter conformed to a double-sigmoid curve. The plot of growth rate vs. time showed four fruit growth stages, SI, SII, SIII, and SIV (Fig. 1a). Transitions between stages occurred at 40, 72, and 100 days AFB.

Free IAA levels, POD, and IAAox activity. The concentration of free IAA 10 days AFB was $123\text{ ng}\cdot\text{g}^{-1}\text{ FW}$, then declined to $46\text{ ng}\cdot\text{g}^{-1}\text{ FW}$ at 16 days AFB (Fig. 1b). Free IAA concentration subsequently increased, peaking at 30 days AFB with $199\text{ ng}\cdot\text{g}^{-1}\text{ FW}$, concurrently with the first exponential phase of fruit growth. On the subsequent sampling dates, the IAA concentration decreased, reaching a minimum of $14\text{ ng}\cdot\text{g}^{-1}\text{ FW}$ at 64 days AFB, when the endocarp lignification was completed. The free IAA concentration increased during the first part of SIII, reached a maximum of $41\text{ ng}\cdot\text{g}^{-1}\text{ FW}$ 85 days AFB, then slightly decreased as ripening approached.

The POD activity (Fig. 1c) was high 10 days AFB, declined until 30 days AFB, then rose until 50 days AFB. The increase in POD activity coincided with the beginning of pit hardening. POD activity declined during SII and remained unchanged through ripening.

The IAAox activity (Fig. 1d) paralleled POD activity peaking at the same date.

Morphological observations. One week AFB, the pubescent epicarp exhibited a unicellular layer organization (Fig. 2a). The mesocarp was compact, with few and small intercellular spaces (Fig. 2b). The endocarp had cells with some electrondense vacuolar inclusions (Fig. 2c) whose phenolic nature was demon-

Fig. 1. (a) 'Redhaven' fruit growth expressed as cheek diameter increase (millimeters/day). The four stages of growth (SI to SIV) were defined according to Chalmers and van den Ende (1975). (b) Free IAA concentration determined by PcAb-ELISA. (c) POD activity. (d) IAAoxidase activity. Vertical bars indicate SE.

strated by the positive reaction to the Reeve test. The cells in the pericarp showed rich cytoplasm that appeared to have undergone recent division. Pericarp meristematic activity remained high during the first 2 weeks AFB, then declined. Four weeks AFB, anticlinal cell divisions were confined to the epicarp (Fig. 2 d and g). The epicarp remained unicellularly layered (Fig. 2d), with cells rich in cytoplasm and organelles (Fig. 2g). The external cell wall was not extensively cutinized. The mesocarp tissues contained roundish cells (Fig. 2 d and e) with poorly differentiated plastids having electrondense stroma, very few thylakoids, and several membrane-bound granular inclusions (Fig. 2h). Starch was absent. The phenolic vacuolar inclusions at the endocarp level were more numerous and larger than those of the mesocarp (Fig. 2 f and i).

Five weeks AFB, during the first part of SI, a dramatic increase in starch grains was observed in the poorly differentiated

Fig. 2. Pericarp 1 week AFB. In all the pericarp regions (ep = epicarp, pm = peripheral mesocarp, dm = deep mesocarp, en = endocarp), cells that have undergone recent divisions can be seen. Large dark inclusions (arrows) are present in the endocarp cells. (sc = seed cavity). Pericarp 4 weeks AFB. (d) Monolayered epicarp (ep) and compact peripheral mesocarp (pm). (e) Deeper mesocarp tissue (dm) showing larger cells with some small intercellular spaces (arrows). (f) Endocarp (en) with small cells having large vacuolar inclusions (arrows). (g) TEM micrograph of epicarp showing anticlinal cell division (arrow). (h) TEM micrograph of a mesocarp plastid with few thylacoids (t) and several granular inclusions (gi). No starch is present. (i) TEM micrograph of endocarp cells. Note the very large phenolic inclusions (arrows) in the vacuoles (v).

chloroplasts of the well-vascularized mesocarp peripheral region (Fig. 3 a and c). The abundance of starch in this cell type was also demonstrated by a positive PAS reaction. Polysaccharide was absent in the deeper and less-vascularized mesocarp tissue (Fig. 3b). Several intercellular spaces were forming in the mesocarp, starting from the cell corners (Fig. 3d) and involving middle lamella digestion. At this stage of development, glandular elements paralleling the vascular tissues were recognizable (Fig. 3e). Their secretion products, filling the central cavity, were PAS positive, indicating polysaccharides. Under TEM (Fig. 3f), in the central cavity of the glandular structures, a mucilage

material with a loose fibrillar net produced by few cell layers surrounding the cavity could be seen. These cells were rich in hypersecreting dictyosomes, with abundant vesicles moving toward and fusing with the plasmalemma to discharge their contents. At the end of SI, the division pattern of the epicarp cells changed, and the cells started to divide in all directions. As a consequence, the epicarp took on a multilayered organization with irregular cell clusters (Fig. 4 a and b). In the mesocarp, where cells were continuously enlarging, the intercellular spaces were numerous and reached the hypodermal region (five to six cell layers), characterized by small cells elongated mainly in

Fig. 3. Epicarp and mesocarp 5 weeks AFB. (a) Monolayered epicarp (ep) and peripheral mesocarp (pm) rich in starch (arrows). (vb = vascular bundles). (b) Deeper mesocarp (dm) with cells devoid of starch. (c) TEM micrograph of a peripheral mesocarp plastid. Large starch granules (s) can be seen in the stroma, although the thylakoidal system (t) is still differentiating (gi = granular inclusions). (d) TEM micrograph showing the formation of an intercellular space among adjacent cells of mesocarp. Note digestion of the middle lamella (ml) at the cell corner (arrow) (cw = cell wall, v = vacuole). (e) A glandular structure (arrow) within the vascular systems (vs) (gc = glandular cavity). (f) TEM micrograph of a glandular structure showing the secreting cells (sec) with hyperactive Golgi bodies (arrow). The loose fibrillar secretion product (double arrows) is visible in cell vesicles, between the plasmalemma and the cell wall, and in the gland's central cavity (gc).

the direction of the fruit surface (Fig. 4a). Starch was abundant and restricted to a peripheral region of the mesocarp, where chloroplasts were present. These organelles showed a rather

differentiated thylakoidal system (Fig. 4c) and few small, membrane-bound granular inclusions. In the inner mesocarp, the cells, voluminous, with large intercellular spaces, contained undiffer-

Fig. 4. Epicarp and mesocarp 6 weeks AFB. (a) The proliferated pubescent epicarp (ep), and the peripheral mesocarp (pm) rich in starch. Note the intercellular spaces (arrows) reaching the hypodermal layers. (b) TEM micrograph showing an original epicarp cell having undergone divisions in different planes. (c) TEM micrograph of a plastid of a peripheral mesocarp cell. Note the development of the thylakoidal system (t) and the few, small, membrane-bound granular inclusions (gi). The organelle contains abundant starch (s). (d) TEM micrograph of a plastid of the deep mesocarp region with a very poorly developed thylakoidal system (t) and large granular inclusions (gi) but devoid of starch. Endocarp lignification 8 weeks AFB. (e) Thickening and lignification of the endocarp (en) cell walls (arrows). No phenolic inclusions are visible in vacuoles. (dm = deep mesocarp). (f) and (g) TEM micrographs showing the thickened cell walls (cw) crossed by cytoplasmic vestures (double arrows), which are connected with plasmodesmata (arrow). The cell content has degenerated.

entiated plastids filled with membrane-bound granular inclusions but devoid of starch (Fig. 4d). Eight weeks AFB, in the middle of SII, the walls of the endocarp cells lignified (Fig. 4e) and the cells evolved to a stone type with numerous simple pits filled with cytoplasmic channels (Fig. 4 f and g). Phenolic inclusions were no longer present in vacuoles.

Thirteen weeks AFB, in the middle of SIII, the mesocarp cells further increased in volume and showed wide intercellular

spaces, except in the hypodermis (Fig. 5a). Starch had completely disappeared from the mesocarp, and the chloroplasts had been converted to chromoplasts, with an electrondense stroma rich in plastoglobules (Fig. 5b).

Fifteen weeks AFB, during SIV, the mesocarp cells were near final size, without any substantial ultrastructural modification. The epicarp was thinner than in the previous developmental stages (Fig. 5c), with a highly cutinized surface (Fig. 5 c and

Fig. 5. Epicarp and mesocarp 13 weeks AFB. (a) In the peripheral mesocarp cells (pm) note the wide intercellular spaces (arrows) and the absence of starch. (b) TEM micrograph of a chromoplast of the peripheral mesocarp with several plastoglobules (arrows). Epicarp 15 weeks AFB. (c) Note the low stainability with the toluidine blue of the swollen epicarp cell walls (arrows). A high degree of cutinization is visible on the outer surface (double arrow). (d) TEM micrograph of the highly cutinized surface (cu) of an epidermal cell. (e) Digested areas (arrow) in a swollen epicarp cell wall.

d) and swollen cell walls. The epicarp was less stainable with toluidine blue (Fig. 5c), and the cell wall showed digested areas (Fig. 5e).

Discussion and Conclusions

Free IAA concentration, determined by PcAb-ELISA, were in agreement with those of Miller et al. (1987) using gas chromatography-mass spectrometry. PcAb-ELISA appeared to be more sensitive than the spectrofluorometric method previously used in the same plant tissue (Vizzotto et al., 1989). Free IAA concentrations of pericarp/mesocarp were high when cell division of the fruit was proceeding rapidly during the first 14 days AFB. As cell division ceased, the free IAA concentration declined. The peaks of hormone concentration found at 30 and 85 days AFB were concurrent with the exponential growth phases (SI and SIII), while the lowest free IAA concentrations were found during SII when the fruit growth rate had lowered and the endocarp lignified. A similar pattern of auxin levels during fruit development was found by Miller et al. (1987), Sanchez-Roland et al. (1990), Vizzotto et al. (1989). These results would suggest an association between free IAA concentration and cell division and enlargement. High activities of POD and IAAox were found during cell division. Subsequently, the activity of both enzymes declined, then increased during SII, peaking at the beginning of the endocarp lignification, followed by a decline near ripening. The same pattern for POD was found by Flurkey and Jen (1978) and Sanchez-Roland et al. (1990), al-

though they followed a different extraction procedure. The increase in POD and IAAox activities concurrent with pit hardening points out a close relationship between the activity of both enzymes and phenolic compound metabolism. In fact, IAA oxidation in vitro is coupled to the co-oxidation of phenols through the formation of H_2O_2 (Grambow and Langenbeck-Schwich, 1983). The same process might occur during pit hardening since the lignin is formed by the action of POD/ H_2O_2 on monolignols or related compounds (Lewis and Yamamoto, 1990).

Ultrastructural observations pointed out that cell divisions in the mesocarp are rapid during the first and 2nd weeks AFB, then cease 28 days AFB. This finding agrees with those obtained in other cultivars of *Persica*, as well as in other *Prunus* spp. (Addoms et al., 1930; Ragland, 1934; Sterling, 1953). Thus, in *Prunus*, cell division accounts only for growth during the early part of SI.

Four weeks AFB, the final cell count in the mesocarp and endocarp appears to be fixed; thus, subsequent growth of the cells is exclusively supported by cell enlargement, which is concurrent with the formation of intercellular spaces, starting from the cell corners. Separation of the cell walls at the middle lamella suggests that the enzymes responsible for the process may be polygalacturonases, as observed in several other fruits (Ben-Arie et al., 1989; Bennett and DellaPenna, 1987) and in the abscission zone of peach fruit (Rascio et al., 1985).

The epicarp retains meristematic activity longer than the other tissues, although, at the end of SI, the model of cell division

changes from anticlinal to multidirectional. Later, the multilayered epicarp undergoes further modifications, such as cell wall swelling and digestion. The cell wall alterations observed at the multilayered epicarp level might correspond to those noticed at the more external layer of the mesocarp in cultivars that retain a monolayered epicarp throughout fruit development (Ben-Arie et al., 1989; King et al., 1987).

The strong cutinization of the epidermal layer occurring during the SIII stage might play a role in limiting the transpiration of the fruit, which in the final growth stages (SIII and SIV) has a high water content.

Although endocarp lignification (pit hardening) occurs during SII, the cells are recognizable from early SI, being smaller and synthesizing a large amount of phenolic compounds. Because phenols are absent in the larger adjacent cells of the mesocarp, the synthesis seems to be specific to the endocarp. This phenomenon might be related to the formation of large lignified parietal masses, as phenols are lignin precursors (Lewis and Yamamoto, 1990). Such a hypothesis is supported by our observations, since the phenolic compounds disappear during endocarp lignification.

In the outer part of the mesocarp, plastids evolving to chloroplasts are present. Their ultrastructure and ontogenetic pattern are characterized by an initial presence of membrane-bound granular inclusions, materials of which may be used in the subsequent building of the thylakoidal system (Casadoro et al., 1977; Rascio et al., 1985). The major localization of starch grains in the peripheral region of the mesocarp, where differentiated chloroplasts are present, would suggest a photosynthetic origin of the polysaccharide. However, the observation that starch accumulates before development of many photosynthetic membranes suggest that starch formation is from photosynthates exported from the leaves. This hypothesis is supported by the high frequency of vascular bundles in the mesocarp peripheral region. In this region, the starch content remains high through SI, then disappears during the following growth phases. This observation agrees with those made on other cultivars by Chalmers and van den Ende (1975) and King et al. (1987). Starch concentrations are inversely related to those observed for sucrose (Pinton et al., 1989). This relationship supports the hypothesis that the different fruit growth stages may be characterized by changes in the cell sugar metabolism and by the sugar transport mechanism.

The mesocarp plastids revert to chromoplasts in the final stages of development and are responsible for the yellowing of the tissue. A secretory system producing mucilages occurs within the mesocarp vascular bundles. To our knowledge, this is the first time that such a structure has been described in *Prunus* fruits.

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